# ANGIOTENSIN CONVERTING ENZYME GENE INSERTION(I)/DELETION(D) POLYMORPHISM AND ITS ASSOCIATION WITH TYPE 2 DIABETICS WITH NEPHROPATHY

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# BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **'ANGIOTENSIN CONVERTING ENZYME GENE INSERTION(I) / DELETION(D) POLYMORPHISM AND ITS ASSOCIATION WITH TYPE 2 DIABETICS WITH NEPHROPATHY'** is the original bonafide work done by **Dr.K.Menaka Shanthi,** Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

**Dr.K.Ramadevi., M.D.,Ph.D., ( Guide ),**
**Professor, Institute of Biochemistry,**
Madras Medical College,
Chennai – 600 003.

**Dr.Pragna B. Dolia., M.D.,**
**Director and Professor,**
Institute of Biochemistry,
Madras Medical College,
Chennai – 600 003.

**Dr.V.Kanagasabai.,M.D.,**
**Dean,**
Government General Hospital
Madras Medical College,
Chennai – 600 003.

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# ABBREVIATION

| | | |
|---|---|---|
| ACE | - | Angiotensin converting enzyme |
| AER | - | Albumin Excretion rate |
| ACR | - | Albumin Creatinine Ratio |
| AGE | - | Advanced glycation end products |
| AGT | - | Angiotensinogen |
| $AGTR_1$ | - | Angiotensin 11 receptor type 1 |
| $AGTR_2$ | - | Angiotensin 11 receptor 2 |
| ANP | - | Atrial Natriuretic Peptide |
| AT II | - | Angiotensin II |
| BP | - | Bloood Pressure |
| CAD | - | Coronary Artery Disease |
| ESRD | - | End Stage Renal Disease |
| GBM | - | Glomerular Basement membrane |
| GFR | - | Glomerular Filtration Rate |
| GLUT | - | Glucose Transporter |
| IL | - | Interleukin |
| LDL | - | Low Density Lipoprotein |
| MCP 1 | - | Monocyte chemoattractant protein 1 |
| TGF-β | - | Transforming growth factor-β |

| | | |
|---|---|---|
| PCR | - | Polymerase Chain Reaction |
| PG | - | Prostaglandin |
| PDGF | - | Platelet Derived Growth factor |
| VEGF | - | Vascular endothelial growth factor |
| PKC | - | Protein Kinase C |
| RAAS | - | Renin Angiotensin Aldosterone System |
| ROS | - | Reactive Oxygen Species |
| eNOS | - | Endothelial Nitric Oxide Synthase |
| DDAH | - | Dimethyl Arginine Dimethyl Amino Hydrolase |
| ADMA | - | Asymmetric Dimethyl Arginine |
| DAG | - | DiAcyl Glycerol |
| NO | - | Nitric oxide |
| PAI1 | - | Plasminogen Activator Inhibitor – 1 |
| DM | - | Diabetes Mellitus |
| EDTA | - | Ethylene Diamine Tetra Acetic Acid |
| DNA | - | Deoxyribonucleic acid |

# INTRODUCTION

# INDEX

# INTRODUCTION

Type 2 diabetes mellitus is a common metabolic disorder having an evident genetic component as shown by the strong familial aggregation and high concordance in twins[1,2]. Poor glycaemic control, duration of the disease and blood pressure (BP) leads to the development of diabetic microvascular complications. Diabetic nephropathy is a serious microvascular complication of diabetes mellitus. The pathogenesis is considered to be multifactorial and genetic, and other factors such as metabolic control and haemodynamic alterations resulting in systemic and intrarenal hypertension might also contribute[3.] However, not all diabetic patients develop renal complications[4]. Clustering of diabetic nephropathy in families[5,6] and the large variation in its prevalence among different diabetic populations suggest the involvement of genetic factors.

The genetic basis of renal complication in diabetes is not clearly understood but many candidate genes have been shown to be associated with diabetic nephropathy[7-9]. Angiotensin converting enzyme(ACE) plays an important role in the renin-angiotensin aldosterone pathway and has been studied extensively as a putative mediator of diabetic nephropathy. This enzyme cleaves the COOH-terminal dipeptide of angiotensin I to produce angiotensin II in liver and inactivates

bradykinin in many tissues[10,11]. Genes encoding components of the renin-angiotensin system (RAS) are suggested as logical susceptibility determinants as angiotensin (Ang) II the final product of the RAS increases intraglomerular capillary pressure causing glomeulrosclerosis[12,13]. Studying the ACE gene is supported by clinical and experimental studies showing that treatment with ACE-inhibitors prevents and reduces progression of diabetic nephropathy[14-16] and suppresses hepatic glucose production in type 2 diabetic patients[17].

ACE gene spans 21 Kb on chromosome 17 and is characterised by the presence (insertion [I]) or absence (deletion [D]) of a 287 bp Alu repeat sequence within intron 16[18]. Although the (I/D) polymorphism is in the intronic region of the ACE gene, this polymorphism is of a functional significance as the ACE levels have been shown to be genetically controlled. Patients homozygous for the II have the lowest ACE levels whilst patients with DD genotype have the highest levels[19,20]. Conflicting findings in various populations have been obtained with regard to the role of ACE (I/D) polymorphism in diabetic nephropathy[21-23] and in type 2 diabetes[23-25]. Ethnic factors might contribute to variability between reports evaluating the role of ACE (I/D) polymorphism. In this study, we intended to determine the distribution of ACE genotypes and its frequency in diabetic patients with nephropathy.

# REVIEW OF LITERATURE

# REVIEW OF LITERATURE

Diabetic nephropathy is a more common cause of chronic kidney disease especially in developing countries.

In diabetic patients the presence of persistant microalbuminuria in the absence of clinical or laboratory evidence of other kidney disease or renal tract disorders is termed as diabetic nephropathy[26].

Diabetic nephropathy is a clinical syndrome characterized by the following:

- Persistent albuminuria (>300 mg/d or >200 μg/min) that is confirmed on at least 2 occasions 3-6 months apart
- Progressive decline in the glomerular filtration rate (GFR)
- Elevated arterial blood pressure

Proteinuria was first recognized in diabetes mellitus in the late 18th century. In the 1930s, Kimmelstiel and Wilson described the classic lesions of nodular glomerulosclerosis in diabetes associated with proteinuria and hypertension.

**Pathological changes in Diabetic nephropathy**:

The nephropathology in diabetic patients is unique to the disease. Thickening of the basement membrane is the first change that can be quantitated. The thickening of the tubular basement membrane parallels the GBM thickening. Afferent and efferent glomerular artery hyalinosis can be detected in early stage of diabetes. This can eventuate in the total replacement of the smooth muscle cells of these small vessels by waxy, homogenous translucent appearing periodic acid Schiff(PAS) positive material consisting of immunoglobulins, complement, fibrinogen, albumin and other plasma proteins[27]. Arteriolar hyalinosis, glomerular capillary subendothelial hyaline and capsular drops along the parietal surface of the bowman's capsule make up the so called exudative lesions of diabetic nephropathy. These lesions are capable of triggering inflammatory cascades as they can fix heterologous complement[26].

Increase in fraction of glomerulus occupied by mesangium can be documented. The diffuse and generalised process of mesangial expansion has been termed as diffuse diabetic glomerulosclerosis (Kimmelsteil Wilson nodular disease) represent areas of marked mesangial expansion, appearing as large round fibrillar mesangial zones

with pallisading mesangial nuclei around periphery of the nodule and extreme compression of associated glomerular capillaries[28].

As disease progresses towards renal insufficiency, more glomeruli become totally sclerosed with complete closure of glomerular capillary lumens. Various studies suggest that the glomerular and interstitial changes of diabetes have somewhat different pathogenetic mechanisms and that advancing interstitial fibrosis generally follows the glomerular processes in diabetes.

The key change in diabetic glomerulopathy is augmentation of extracellular matrix. The earliest morphologic abnormality in diabetic nephropathy is the thickening of the GBM and expansion of the mesangium due to accumulation of extracellular matrix. The severity of diabetic glomerulopathy is estimated by the thickness of the peripheral basement membrane and mesangium and matrix expressed as a fraction of appropriate spaces (eg, volume fraction of mesangium/glomerulus, matrix/mesangium, or matrix/glomerulus).

The glomeruli and kidneys are typically normal or increased in size initially, thus distinguishing diabetic nephropathy from most other forms of chronic renal insufficiency, wherein renal size is reduced.

**Pathogenesis of diabetic nephropathy:**

The simple scheme for the pathogenesis of diabetic nephropathy is shown in figures 1, 2 and 3.

Since nephropathy does not develop in all diabetic patients, genetic and hemodynamic factors in addition to hyperglycemia must be operative in these patients at risk of development of diabetic nephropathy.

Factors predicting high risk for development of diabetic nephropathy include:

1. Poor glycemic control.
2. Hemodynamic injury (systemic and intravascular glomerular hypertension).
3. Familial and genetic factors.
4. Dyslipidemia

**1. Hyperglycemia:**

A host of metabolic consequences of hyperglycemia have been described as an explanation for renal lesions. Hyperglycemia is a crucial

**Figure-1**

**INTERACTION OF HEMODYNAMIC AND METABOLIC PATHWAY, CYTOKINES AND INTRACELLULAR SIGNALLING MOLECULES MEDIATING DIABETIC NEPHROPATHY**

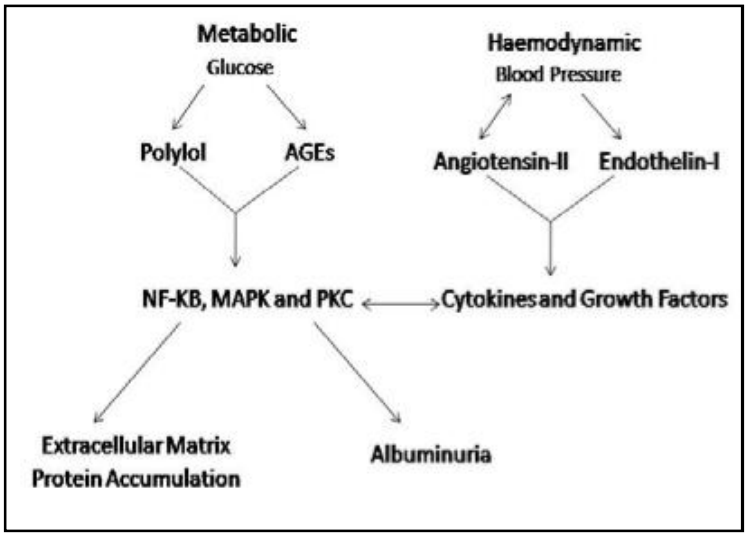
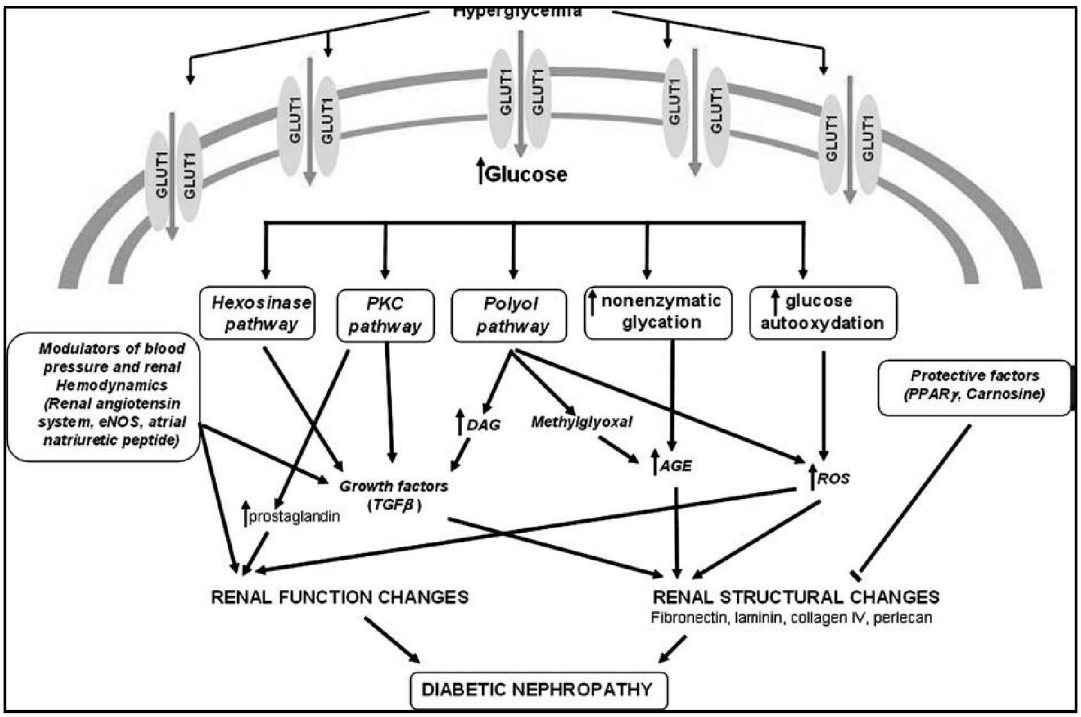



**Figure-2**

**METABOLIC AND HEMODYNAMIC FACTORS IN DIABETIC NEPHROPATHY**



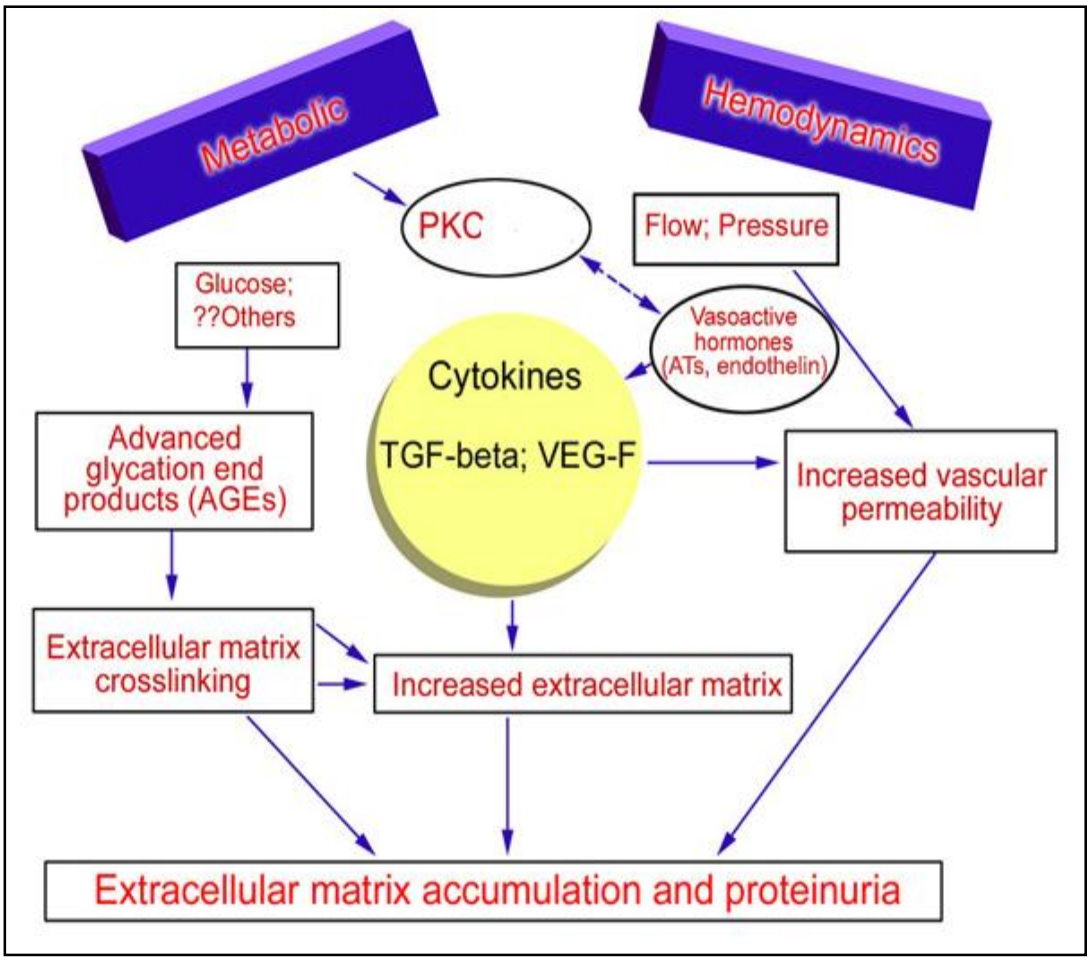
factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity.

Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening. In vitro studies have demonstrated that hyperglycemia is associated with increased mesangial cell matrix production[29,30] and mesangial cell apoptosis[31,32]. Mesangial cell expansion seems to be mediated in part by an increase in the mesangial cell glucose concentration, since similar changes in mesangial function can be induced in a normal glucose milieu by overexpression of glucose transporters, such as GLUT1 and GLUT4, thereby increasing glucose entry into the cells[30].

Hyperglycemia might also upregulate VEGF expression in podocytes[33], which could markedly increase vascular permeability[34]. Hyperglycemia, however, does not account fully for the risk of diabetic nephropathy, as shown by studies in which kidneys from nondiabetic donors were transplanted into patients with diabetes and nephropathy

**Figure 3**

**SCHEMATIC REPRESENTATION OF METABOLIC AND HEMODYNAMIC FACTORS IN DIABETIC NEPHROPATHY**



developed irrespective of the glucose control[35]. Hyperglycemia might, therefore, be necessary for but not sufficient to cause renal damage.

Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway[36,37]. Oxidative stress seems to be a theme common to all three pathways[38].

These include

- **Polyol pathway:**

Hyperglycemia has been proposed to activate polyol pathway in insulin insensitive tissues. This is suspected to mediate neuropathy, retinopathy and perhaps nephropathy. The mechanism proposed is disorder in the cellular myoinositol metabolism due to increased flux of glucose through the polyol pathway. In addition the pathway may also provide additional reactive substrates for non enzymatic reactions involving the proteins which are responsible for vascular complications[39].

In addition, the increased flux through polyol pathway results in increased ratio of NADH:$NAD^+$ which appears to be important in the stimulation of denovo synthesis of diacyl glycerol and subsequently activation of PKC.

Clinical trials of aldose reductase inhibitors have not shown beneficial effects in reducing microalbuminuria in humans; however, research continues in this arena.

- **Non – enzymatic glycation:**

The advanced glycation end products (AGE) modify the ligands on extracellular components and interact with cellular receptors to alter intracellular function. AGEs likely upregulate a host of genes that can adversely affect protein structure and function. AGEs also cause increased cross-linking and reduced breakdown of the thickened glomerular basement membrane constituents and enhanced binding of circulating plasma proteins and activation of cells, particularly macrophages. AGE modified LDL also has impaired clearance[40].

AGEs have been shown to correlate with microalbuminuria in diabetic patients. In a study of low- and high-molecular-weight AGEs in subjects with and without diabetes, AGE content in arterial wall

collagen was fourfold higher in diabetes[41]. Diabetic patients with ESRD had twice as much tissue AGE as patients without renal disease. Circulating AGEs were elevated in patients with diabetes compared to those without diabetes, and the levels correlated directly with creatinine.

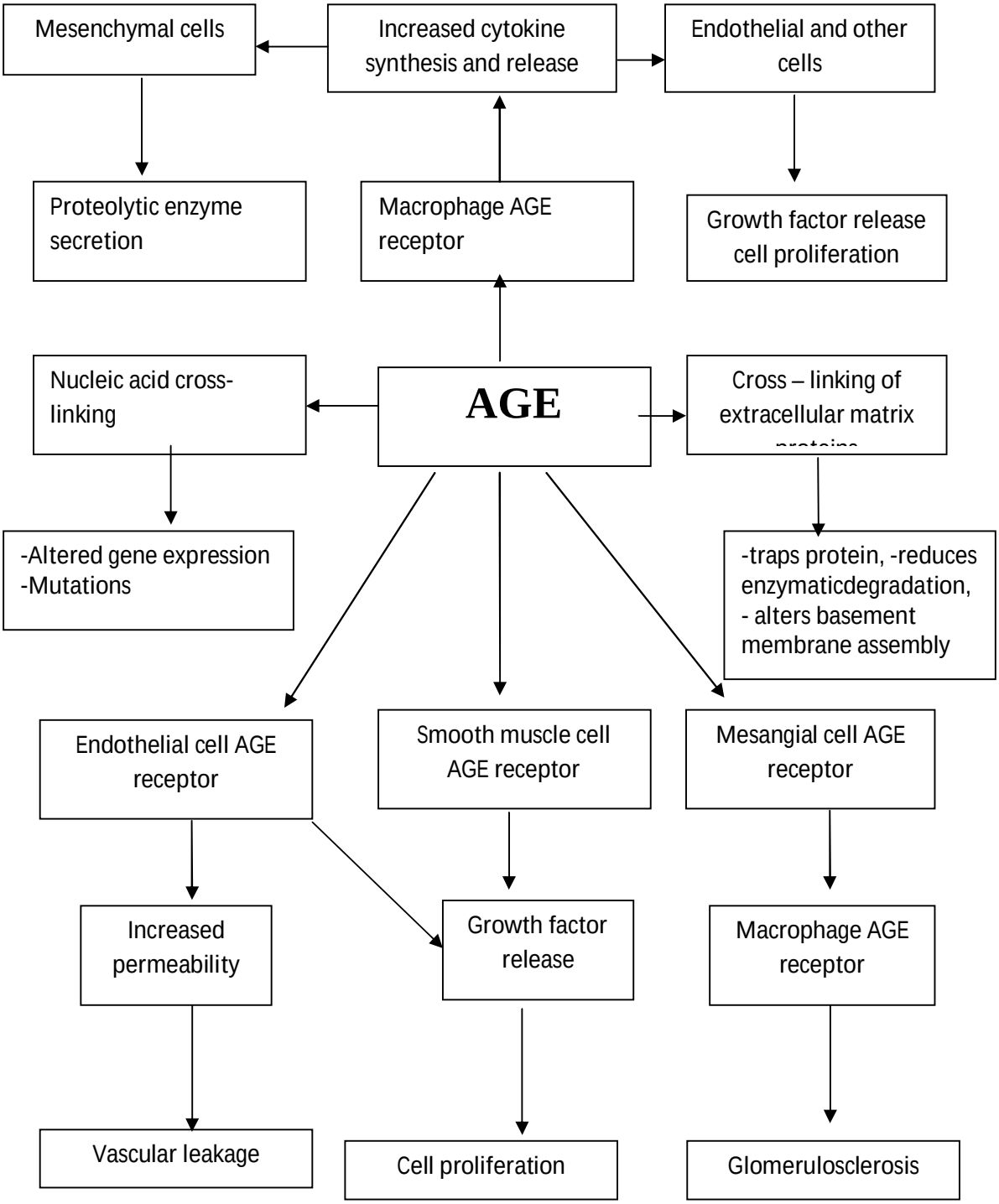
Novel drugs such as Aminoguanidine are being evaluated for their therapeutic benefit in the management of diabetic nephropathy as these compounds bind preferentially to reactive precursors of AGEs and form unreactive substituted products that can no longer form cross-links[42]. Schematic representation of the possible mechanisms by which advanced glycation end – products (AGEs) and AGE receptors may contribute to the pathogenesis of diabetic nephropathy is illustrated in chart 1.

- **Hormones and cytokines:**

**TGF-β:** High ambient glucose seems to promote renal cellular hypertrophy and matrix accumulation via increased expression of TGF-β by the kidney (ie. Glomrular and tubular cells). It regulates cell hypertrophy and the production of extracellular matrix elements such as collagen, fibronectin, tenascin, laminins and proteoglycans. They also promote cell matrix interactions and matrix accumulation. They regulate

**Chart- I**

**AGE AND ITS RELATION WITH DIABETIC NEPHROPATHY**



extra cellular matrix components by upregulating the synthesis of protease inhibitors and downregulating the synthesis of matrix degrading proteases. Hyperglycemia also has been observed to upregulating signalling receptors for TGF-β in kidney cells[43].

Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy, specifically interleukin 1 (IL-1), IL-6 and IL-18 and tumor necrosis factor. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms. In addition, raised levels of several of these cytokines in serum and urine correlate with progression of nephropathy, indicated by increased urinary albumin excretion[44].

- **PKC**:

Hyperglycemia resulting in increased functioning of polyol pathway results in increased ratio of $NADH/NAD^+$ , this results in shunting of some glycolytic intermediates into phsphatidic acid and diacylglycerol[45]. This increased synthesis of diacylglycerol causes activation of diacylglycerol causes activation of PKC resulting in increased matrix production. Activation of this enzyme leads to

increased secretion of vasodilatory prostanoids, which contributes to glomerular hyperfiltration.

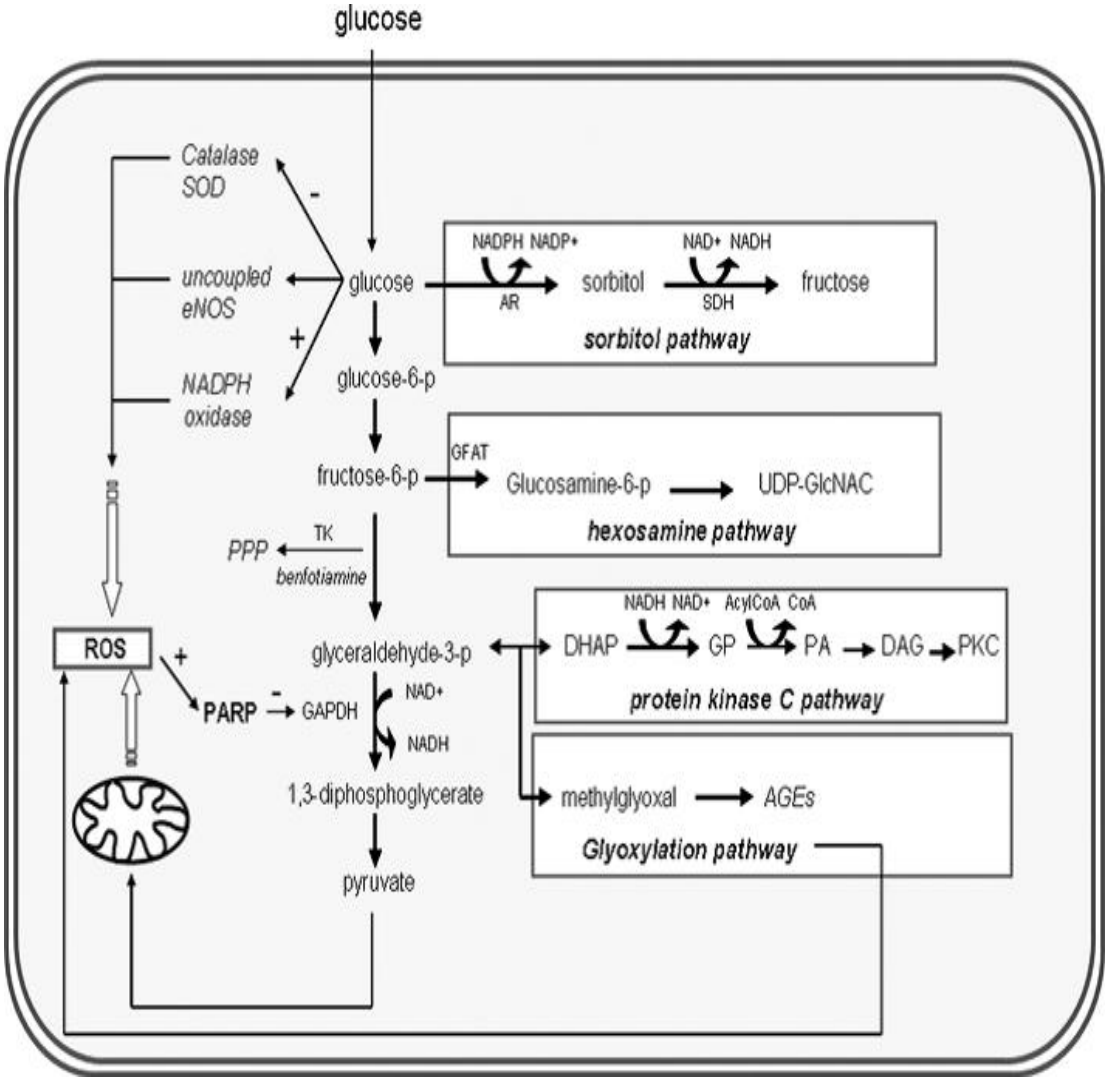
PKC leads to increased production of Jun/Fos (AP 1) transcription factor complex. This factor binds to and activates several gene promoters including growth factors and matrix molecules. PKC may also be involved in TGF-β overexpression – as a consensus AP1 binding site and other glucose responsive elements have been identified in the promoter region of the TGF-β1 gene. By activation of TGF-β1, PKC increases production of extracellular matrix by mesangial cells[46]. PKC activation induces the activity of mitogen-activated protein kinases (MAPK) in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. The coactivation of PKC and MAPK in the presence of high glucose concentrations indicates that these two families of enzymes are linked[47]. Fig-4 shows pathogenesis of diabetic nephropathy.

- **Angiotensin II:**

In diabetic nephropathy, the activation of the local renin-angiotensin system occurs in the proximal tubular epithelial cells, mesangial cells, and podocytes. Angiotensin II (ATII) itself contributes

**Figure-4**

**METABOLIC PATHWAYS AND ROS IN PATHOGENESIS OF DIABETIC NEPHROPATHY**



to the progression of diabetic nephropathy. ATII is stimulated in diabetes despite the high-volume state typically seen with the disease, and the intrarenal level of ATII is typically high, even in the face of lower systemic concentrations. Ang II may underlie the altered renal hemodynamics in diabetics. ATII preferentially constricts the efferent arteriole in the glomerulus, leading to higher glomerular capillary pressures.

Ang II also has additional non-hemodynamic effects, as renal growth factor and a profibrinogenic agent that stimulates ,matrix synthesis, at least in part due to stimulation of TGF-β activity[48] and other growth factors. The endothelial cells contain ACE, which converts Ang I to Ang II.

NO has been shown to downregulate the synthesis of ACE[49] in the endothelium, as well as Ang II type 1 receptors (AT1) in vascular smooth muscle cells, thus having the potential to decrease Ang II production and action[50,51]. ACE inhibitors not only decrease Ang II synthesis but prevent the degradation of bradykinin, one of the most important physiological molecules involved in the release of NO[52]. The main subtypes of Ang II receptors are AT1 and AT2[53]. AT1 mediates the vasoconstrictor effect of Ang II and mediates the Ang II–induced

growth in cardiovascular and renal tissue[52]. NO can downregulate AT1 receptors in vascular tissue[50] and mitigate the actions of Ang II[54]. AT2 actions are less well understood. Figure -5 showing NO and AT II relationship in diabetic nephropathy.

The fact that ACE inhibitor are renoprotective, strongly suggests a role of increasing systemic and intrarenal production of Ang II in diabetic nephropathy.

- **Eicosanoids:**

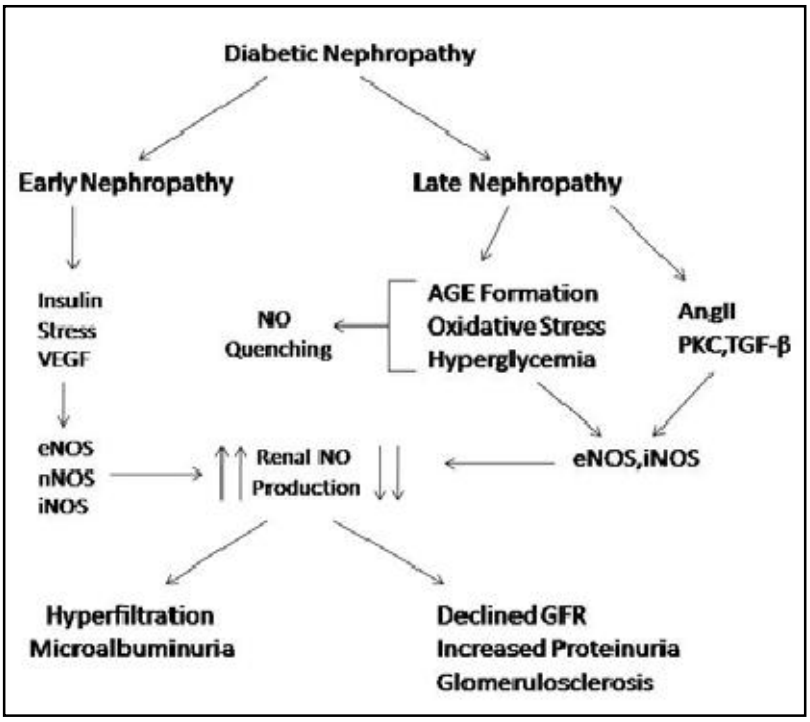
Vasodilatory prostanoids (PGE2, PGI2) contribute to early glomerular hypertension. There is also evidence of increase in thromboxane (TXA2) generation, correlating with the development of proteinuria. In vitro studies have demonstrated increased fibronectin production by mesangial cells following addition of thromboxane analogues. It is also known to stimulate TGF-β production in mesangial cells.

- **Other mediators:**

  - ✓ Nitric oxide appears to be a mediator of glomerular hyperfilteration.

**Figure – 5**

**NITRIC OXIDE AND ANGIOTENSIN II IN DIABETIC NEPHROPATHY**



- ✓ Change in cytosolic calcium (increase) has been shown to participate in the genesis of some of the diabetes complications ie. Leukocyte and B cell dysfunction[57].

2. **Glomeular hemodynamics in Diabetic nephropathy:**

Patients with diabetes tend to have a greater GFR than nondiabetics. This hyperfiltration in early diabetes results from increase in blood flow and a concomitant increase in glomerular capillary hydraulic pressure[58]. Possible factors responsible for this may be increased vasodilatory prostanoids, nitric oxide and humoral vasodilators (glucagon and ANP)[59]. Increased sodium reabsorption in proximal tubules leads to reduced sodium delivery to macula densa and alters tubuloglomerular feedback. In addition, the glomerular capillary surface area available for filteration is increased in diabetics and contributes to increased GFR.

The early increase in glomerular blood flow and glomerular capillary pressure enhances the shear stress effect on the glomerular capillary wall and the mesangium. The stess may lead to endothelial dysfunction, which translates into alterations in GBM structure as well as mesangial cell dysfunction. Intraglomerular hypertension can

- ✓ IGF-1 is implicated in diabetic glomerular hypertrophy.

- ✓ Elevated PDGF β and interleukin expression has been observed in the glomeruli of diabetic rats.

- ✓ Hyperglycemia may also cause damage via increased oxidative stress by promoting reactive oxygen species production as well as attenuating free radical scavenging molecules. Hyperglycemia specifically induces oxidative stress, even before diabetes becomes clinically apparent. Concentrations of markers of DNA damage induced by reactive oxygen species are higher in patients with more-severe nephropathy (i.e. proteinuria versus microalbuminuria). Furthermore, histological analysis of human kidney biopsy specimens has detected products of glyco-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without diabetes[55,56].

increase the macromolecular trafficking across the mesangium and that can provoke the mesangial cells to increase their production of mesangial matrix components[60].

Mesangial expansion may be a specific example of how renal ie particularly glomerular hypertrophy can be disadvantageous. A link may be present between the intraglomerular hypertension of diabetic nephropathy and stretch induced production of prosclerotic cytokine – transforming growth factor beta (TGF-β)[61].

According to Steno hypothesis, all the diabetic microvascular complications are manifestations of increased vascular permeability and endothelial damage in various organs. This view says that glomerular endothelial dysfunction with the ensuing disturbance in glomerular permeability is just one facet of generalised endothelial dysfunction[62].

**3. Familial and genetic factors:**

Genetic susceptibility to diabetic nephropathy has been suggested on the basis of the observation that only one-third of the individuals with diabetes ever develop nephropathy, irrespective of glycemic control. There has also been strong clustering of diabetic nephropathy in families.

Polymorphism of ACE gene has received lots of attention in the above respect. Insertion/Deletion polymorphism in the ACE gene have been found to predict the severity of disease and rate of progression[63]. Polymorphism of apo E has also been found to be associated with diabetic nephropathy[64].

**4. Dyslipidemia:**

Dyslipidemia has also been found to be associated with microalbuminuria in many studies[65-67].

**Proteinuria:**

In addition to the risk factors mentioned above , proteinuria itself causes progression of renal damage. Increased glomerular permeability will allow plasma proteins to escape into the urine. Some of these proteins will be taken up by the proximal tubular cells, which can initiate an inflammatory response that contributes to interstitial scarring eventually leading to fibrosis. Tubulointerstitial fibrosis is seen in advanced stages of diabetic nephropathy and is a better predictor of renal failure than glomerular sclerosis. The excessive tubular reabsorption of proteins induces the release of vasoactive and inflammatory cytokines such as Endothelin1, Osteopontin and monocyte

chemoattractant protein 1 (MCP 1). These factors in turn cause overexpression of proinflammatory and fibrotic cytokines and infiltration of mononuclear cells resulting in injury to tubulointerstitium[68]. There is an epithelial-mesenchymal transition that takes place in the tubules, with proximal tubular cell conversion to fibroblast-like cells. These cells can then migrate into the interstitium and produce collagen and fibronectin.

Even the beneficial effects of ACE inhibitors in diabetic renal disease, reflects their antiproteinuric action in addition to reduction of Ang II mediated effects on growth factor activation and glomerular hemodynamics[68].

The present concensus is that all the above mentioned risk factors contribute to chronic low grade inflammation, which is present in the diabetic state. This is said to be responsible for endothelial dysfunction resulting in altered basement membrane synthesis, contributing to arterial stiffness and increased vascular permability.

**Clinical stages of diabetic nephropathy:**

20%-30% of patients with Type 2 Diabetes will suffer from Diabetic Nephropathy, which means that one third of Diabetic patients

develop into Diabetic Nephropathy. Diabetic nephropathy can be characterized into different clinical stages which differ with respect to renal hemodynamics, systemic BP and urinary albumin excretion rate(AER).

**Stage 1: Glomerular ultrafiltration period**:

In this period, glomerular filtration rate is very high and patients have no obvious symptoms but manifests with renal hypertrophy, elevated renal blood flow and increased GFR. AER is typically within normal range. The histopathological changes are potentially reversible with insulin therapy and stringent glycemic control. Diabetic patients with extremely high GFR (above 150-170 mL/min) during this stage are at increased risk of future development of diabetic nephropathy. Nephropathy is difficult to perceive in this period, so Diabetic patients must make urine check during less than 6 months.

**Stage 2 : Slow development period/Clinical latency/Incipient stage:**

The urine microalbuminuria excretion rate increases after doing exercises and it becomes normal after rest in this period of Diabetic Nephropathy. Glomerular structure changes in thickening of glomerular basement membrane and increase of mesentery matrixes.

**Stage 3 : Early Diabetic Nephropathy period/Microalbuminuria:**

Typically occurs 5-15 yrs of onset of diabetes. In this period, blood pressure of patients raised by the influence of Diabetic Nephropathy and urine microalbuminuria excretion can be decreased with the falling of blood pressure. Glomerular structure changes obviously in thickening of glomerular basement membrane and increase of mesentery matrixes. Histologically diffuse or nodular glomerulosclerosis as well as early signs of arteriosclerosis and tubulointerstitial fibrosis is seen. GFR begins to decline. Serum creatinine may still be in the normal range.

**Stage 4 : Clinical Diabetic Nephropathy period/Macroalbuminuria:**

A great deal of albumins appears and patients who have urine protein with more than 3.5g/24h may have the symptoms like hypoalbuminemia, edema and high blood pressure always with nitrogen retention and Diabetic eye disease.

**Stage 5 : End-stage Renal Failure period:**

Urine protein excretion decreased with the failing of the glomerulus and glomerular filtration rate is less than 10ml/min. In this

period of Diabetic Nephropathy, patients present high blood pressure, hypoalbuminemia, edema, increasing of serum creatinine and urea nitrogen, anorexia, nausea and vomiting, anemia and some other symptoms. Secondary Uremia neuropathy and Cardiomyopathy can be caused by End-stage Renal Failure.

**Clinical diagnosis :**

Among the earliest changes demonstrable in diabetic nephropathy is glomerular hyperperfusion. This is accompanied by microalbuminuria, which serves as a sensitive early indicator of adverse effects of diabetes on the kidney and is a powerful predictor of the subsequent course. Clinically, the most important screening tool for identifying early nephropathy is detection of microalbuminuria. The prevalence of microalbuminuria in patients with diabetes is 10-30%[69].

Microalbuminuria is also a powerful predictor of cardiovascular disease in both type 1 and type 2 diabetes. In type 2 diabetes, diabetes has been present for several years by the time it is diagnosed so annual microalbuminuria screening should begin at the time of diagnosis. Several methods are available for screening and give comparable results. The gold standard is the 24-hour urine collection (normal

albumin excretion <30 mg per 24 hours), which, if accompanied by serum and urine creatinine, also allows calculation of creatinine clearance rate and serves as a reference for future comparison.

Somewhat more convenient for the patient is the albumin/creatinine ratio on a spot urine sample (normal <30 mg albumin per g creatinine) or albumin excretion rate in a timed specimen (4 hours or overnight, normal <20 mg albumin per min). Measuring albumin without relating it to a duration of collection or creatinine concentration is less sensitive and specific because of dilution variability. Several methods are available for screening and give comparable results.

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There is a diurnal variation in urinary protein excretion, with less glomerular protein leak during nighttime and recumbency. Thus, measurement on collections of less than 24 hours may show the effect of time of day. There is also considerable variation from day to day, so three collections should be carried out over a 6-month period with the designation of microalbuminuria reserved for those with elevations on two out of three measurements.

Furthermore, screening should avoid other factors that may temporarily induce albuminuria, such as poor glycemic control, exercise, fever, urinary or systemic infections, and marked hypertension.

Both glycemic control and rigorous control of blood pressure have significant impact on prevention and progression of diabetic nephropathy[70]. Identification of patients with microalbuminuria selects a population of patients with increased mortality. Studies in both type 1 and type 2 patients show that the use of ACE inhibitors leads to decreased albumin excretion and may postpone or even prevent overt nephropathy[71].

## ANGIOTENSIN-CONVERTING ENZYME (ACE)

Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. Angiotensinogen is converted to angiotensin I by stimulation of renin. ACE then converts angiotensin I to angiotensin II, the main active product of the renin–angiotensin–aldosterone system (RAAS).

The human ACE gene is located on chromosome 17q23[72], and includes 26 exons. The coding sequence codes for a 1306 amino acid protein, including a single peptide. The gene product, ACE, is composed of 2 homologous domains with 2 active sites[73]. The ACE gene product ACE converts angiotensin I to angiotensin II, and is involved in the degradation of bradykinin. Bradykinin acts as a potent stimulator of nitric oxide (NO) release. NO plays a crucial role in protecting the endothelium from injury. Furthermore, it has been reported that hypertensive effects are mediated in a bradykinin-dependent manner.

The ACE gene contains a polymorphism in the form of either insertion (I) or deletion (D) of a 287 base pair Alu repetitive sequence in intron 16[74]. This polymorphism is shown to be associated with the interpersonal variability of ACE levels in circulating blood[18]. The

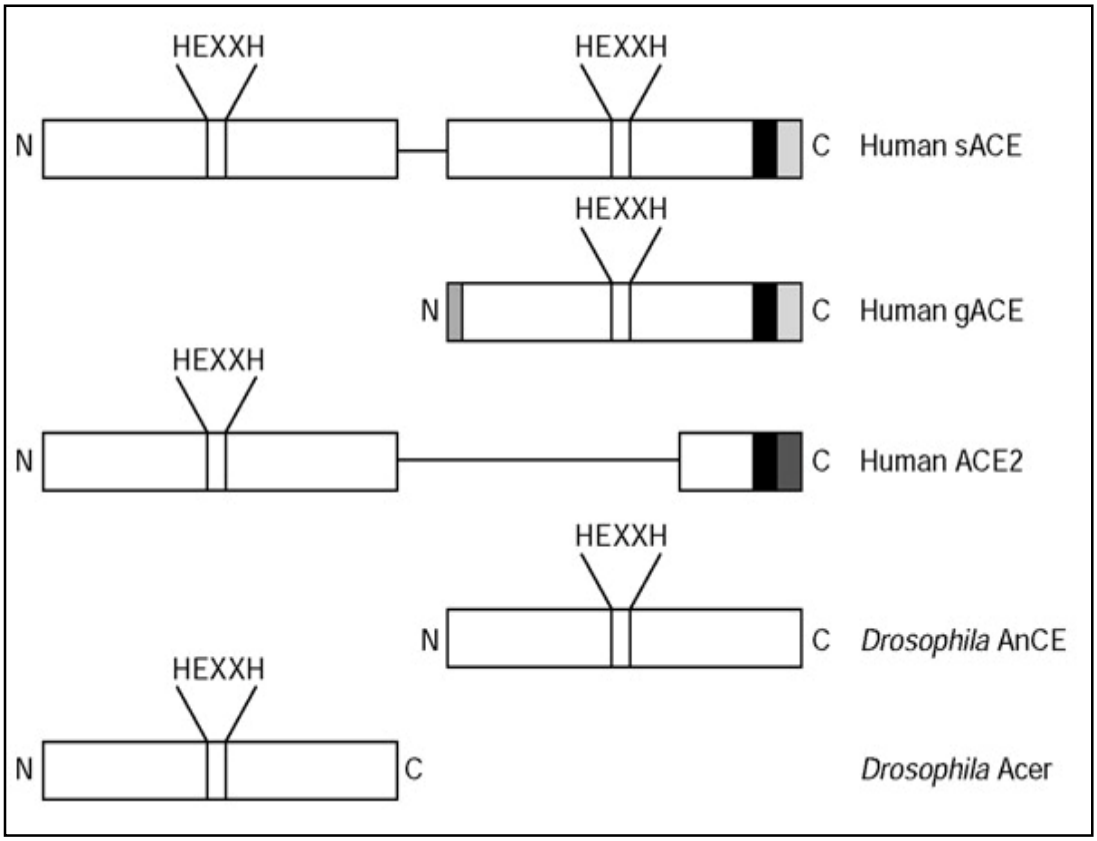



**Figure 6**

Schematic representation of the primary structure of several members of the ACE protein family. The locations of the active-site zinc-binding motifs are indicated by HEXXH; transmembrane domains are in black. The sequence of gACE is identical to that of the C domain of sACE, except for its first 36 residues. Human gACE and sACE have the same carboxy-terminal transmembrane and cytosolic sequences, whereas ACE2 has a distinct transmembrane and cytosolic sequence. Neither of the *Drosophila* ACEs, AnCE and Acer, has a membrane-anchoring sequence. Dimensions are not to scale. N, amino terminus; C, carboxyl terminus. The single lines are regions of sequence with no similarity to other proteins. The carboxyl end of ACE2 is homologous to collectin, a non-enzymatic protein associated with renal injury.

deletion allele at this gene site is associated with increased plasma ACE activity. An increased risk of progression of renal disease associated with the

ACE-D allele has been reported in some populations with renal disease[75,76] but not in others[77,78]. A genetic predisposition to diabetic nephropathy based on the DD genotype has been reported in several studies[22,79,80] but remains controversial[81]

**Structure of ACE:**

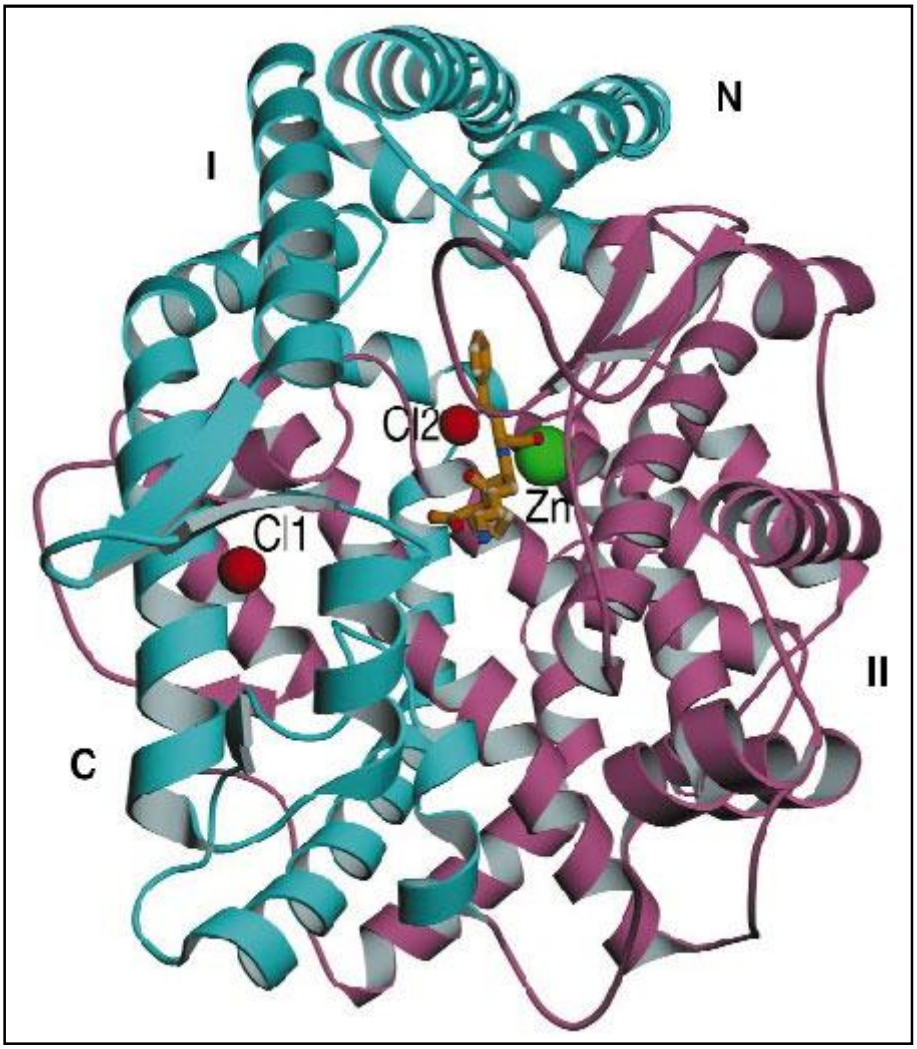
There are two forms of ACE (Fig-6) in humans, encoded by a single gene located on chromosome 17 at q23; it is 21 kb in length and contains 26 exons and 25 introns. The longer form, known as somatic ACE (sACE), is transcribed from exons 1-12 and 14-26, whereas the shorter form, known as germinal or testicular ACE (gACE), is transcribed from exons 13-26. The promoter for s ACE is in the 5' flanking region of the first exon, whereas that for g ACE is located within intron 12[82]. Somatic ACE consists of an intracellular domain, a transmembrane domain and two similar extracellular domains, the amino or N domain and the carboxy or C domain.

The structure of the ACE gene is the result of gene duplication; the N and C domains are similar in sequence, and the homologous exons encoding the N and C domains (exons 4-11 and 17-24, respectively) are very similar in size and have similar codon phases at exon-intron boundaries. Each of the domains contains a catalytically active site characterized by a consensus zinc-binding motif (HEXXH in the single-letter amino-acid code, where X is any amino acid) and a glutamine nearer the carboxyl terminus that also binds zinc; ACE and its homologs therefore make up the M2 gluzincin family[83].

The X-ray structure(Fig-7) of testicular ACE, and its complex with the widely used ACE inhibitor lisinopril, at 2.0 Å resolution has been elucidated. This structure was determined using the anomalous scattering of the bound Zn atom at beam line BM14 of the ESRF.The three-dimensional structure reveales that ACE is composed of a-helices for the most part, and incorporates a zinc ion and two chloride ions. In fact it bears little resemblance to carboxypeptidase A except in the active site zinc-binding motif. Instead, it resembles rat neurolysin and *Pyrococcus furiosus* carboxypeptidase, despite sharing little amino-acid sequence similarity with these two proteins. This similarity extends to the active site, which consists of a deep, narrow channel that divides the

**Figure-7**

**STRUCTURE OF ACE**



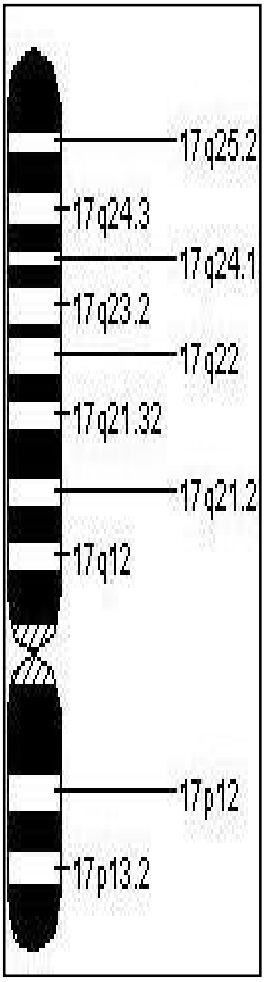
molecule into two subdomains. On top of the molecule is an amino-terminal 'lid', which seems to allow only small peptide substrates (2530 amino acids) access to the active site cleft this accounts for the inability of ACE to hydrolyse large, folded substrates.

**The Genes of the RAS:**

The genes encoding components of the renin-angiotensin system (RAS) present attractive candidates for diabetic nephropathy. The RAS gene system comprises the renin, angiotensinogen (AGT), angiotensin I-converting enzyme (ACE), and angiotensin II receptor types 1 and 2 ($AGTR_1$, $AGTR_2$) genes. The renin gene maps to chromosome 1q32, spans approximately 12 kb, and comprises 10 exons and nine introns[84,85]. The angiotensinogen gene maps to chromosome 1q42–43, spans approximately 13 kb, and comprises five exons and four introns[86,87]; exons 1 and 5 encode for the 5' and 3' untranslated regions of mRNA, respectively. The ACE gene maps to chromosome 17q23(Fig-8), spans 21 kb, and comprises 26 exons and 25 introns[88,89]. The two major species of ACE mRNA are a 4.3-kb endothelial-type mRNA (transcription encompassing exons 1 to 26, excluding exon 13) and a 3-kb testicular type ACE mRNA (transcription encompassing exons 13 to 26). Exon 26 encodes for the functionally important membrane-

**Figure-8**

**CHROMOSOMAL LOCALISATION OF ACE**



anchoring domain of the ACE protein. The endothelial type of ACE mRNA is found not only in endothelial cells, but also in epithelial cells. The angiotensin II receptor type 1 gene maps to chromosome 3 and the angiotensin II receptor type 2 gene maps to chromosome X[90-92].

**Circulating RAS Components**

RAS functions as an endocrine system. The renin gene is expressed primarily in the juxtaglomerular cells of the kidney, where renin is synthesized, stored, and released into the circulation. Prorenin is cleaved to form renin, which is stored in tissue granules until it is released in response to specific secretagogues. Secretion of renin from the kidneys is controlled by several factors. The macula densa are a specialized group of distal convoluted tubular cells that act as chemoreceptors for sodium and chloride levels in the distal tubule. Sodium retention increases blood volume, which is followed by an increase in blood pressure. This increase in blood pressure activates a negative feedback regulation of the juxtaglomerular cells in the kidney, which sense renal perfusion pressure and renin production are inhibited. Renin secretion is autonomically modulated via sympathetic innervation of the renal tubules and arterioles.

**Figure-9**

**ACTIONS OF ACE**

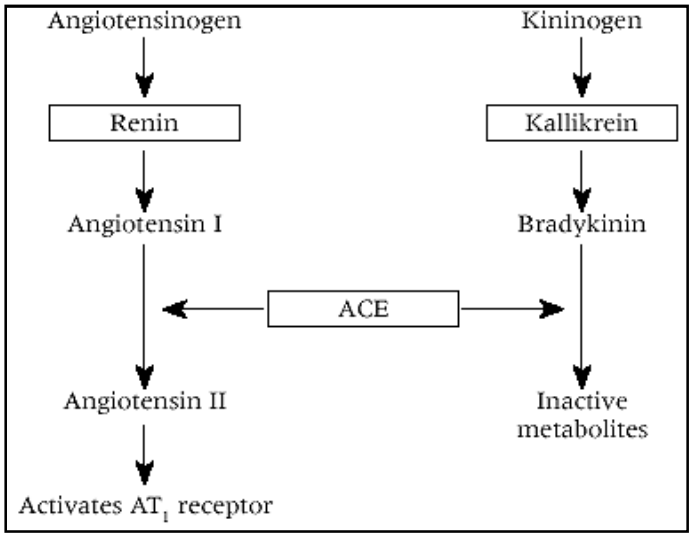
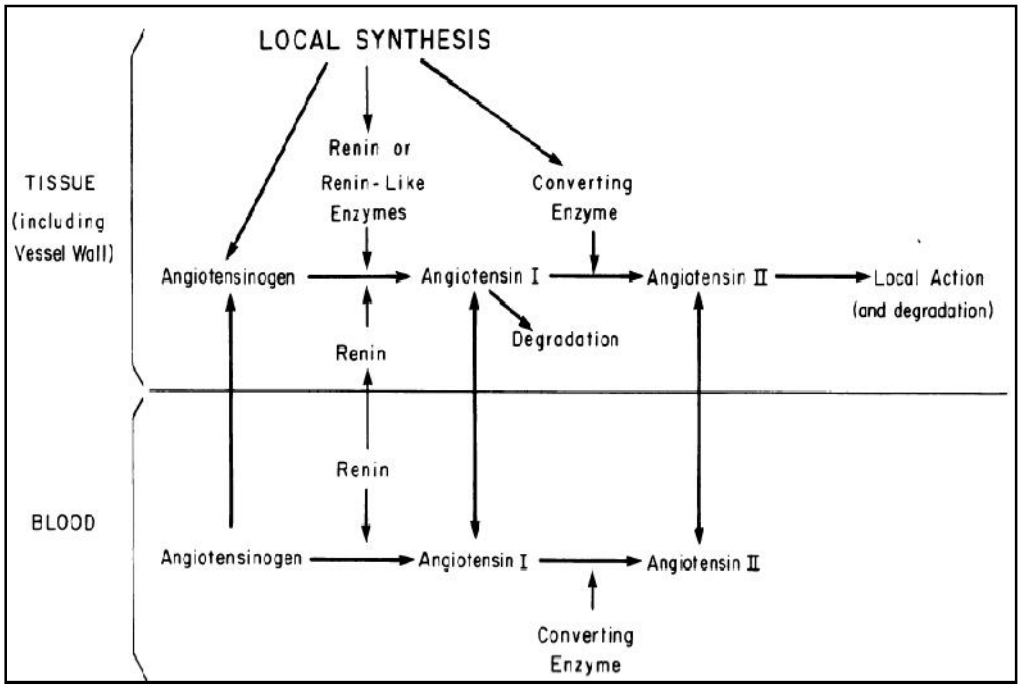



**Figure-10**

**CIRCULATING AND TISSUE FORMS OF ACE**



Circulating renin catalyzes the angiotensinogen-to-angiotensin I conversion (Fig-9). The angiotensinogen gene is expressed in the liver, the site of AGT synthesis and release into the circulation. The angiotensin I (Ang I) generated by renin activity is a vasoinactive decapeptide. Conversion of angiotensin I to angiotensin II (Ang II) is the key reaction in the RAS pathway, generating the effector of the system, Ang II, a potent vasoconstrictor(Fig-10). The reaction is catalyzed by ACE (kininase II; EC 3.4.15.1), a zinc metallopeptidase member of the Alu family that functions as a dipeptidyl carboxypeptidase (DCP1). The mechanisms controlling the circulating ACE levels are less clear than those for renin. The most likely genetic control is at the level of transcription and would involve linkage disequilibrium with regulatory elements of the ACE gene. Once the protein is translated and bound to the cell membrane, release would require cleavage of the hydrophobic bonds that anchor the protein to the membrane. ACE cleaves the C-terminal His-Leu dipeptide from Ang I, generating the vasoactive octapeptide Ang II[93]. Further conversion of Ang II to Ang III is possible by cleavage of the aspartic acid from position 1 of the octapeptide; however, the generated Ang III is less potent as a vasoconstrictor, compared to Ang II[93]. Circulating ACE is found in biological fluids,

such as plasma, amniotic and seminal fluids, and originates from endothelial cells.

ACE also acts as a protease on bradykinin, cleaving the C-terminal Phe-Arg dipeptide, with the net effect of inactivating this vasodilator. Therefore, ACE enzymatic activity will result in a double effect: activation of a vasoconstrictor/pressor (Ang II) agent and inactivation of a vasodilator agent (bradykinin). Ang II is also an aldosterone-stimulating peptide. Aldosterone promotes depletion of potassium while promoting the retention of sodium and water; therefore Ang II exerts a negative feedback on rennin production due to volume expansion and/or to a direct effect on juxtaglomerular cells.

**Tissue RAS Components**

RAS also functions as a paracrine system. Ang II is demonstrated to be produced in multiple target organs by local RAS pathways. However, under normal conditions the renin responsible for local Ang I generation appears to derive from circulation, being of renal origin. The key component of the tissue RAS, as in circulating RAS, is ACE. At the cellular level, the ACE molecule projects into the extracellular space and is anchored to the plasma membrane by the C-terminal hydrophobic

region that spans the membrane and ends in a short cytoplasmic tail. Ang II generated by ACE activity exerts its effects by binding to angiotensin II receptors, type 1 and type 2; $AGTR_1$ is the major mediator of physiological effects of Ang II (vasoconstriction, hypertrophy,catecholamine liberation at sympathetic nerve endings). Both $AGTR_1$ and $AGTR_2$ are transmembrane receptors, comprising seven membrane-spanning domains, and are coupled to G-proteins.

The ACE gene polymorphism was first reported by Rigat et al in a study that addressed the role of the ACE gene in the genetic control of plasma ACE levels[18]. Normally, plasma ACE levels show marked interindividual variation but appear to be remarkably stable when measured repeatedly in the same subject. The normal ranges for plasma ACE levels and the units of measurement depend on the detection method used. Rigat et al used direct radioimmunoassay measurement of the enzyme (in µg/L); subsequently, functional assays using spectrophotometric measurements (in U/L) have been used. Reference ranges for each method must be established in the testing laboratory.

A current and widely used method is a spectrophotometric method using the synthetic tripeptide substrate *N*-[3-(2-furyl)acryloyl]-L-phenyl-alanylglycylglycine (FAPGG). The normal ranges are age-

dependent and vary widely in adults (8–52 U/L). The polymorphism discovered by Rigat et al is of the insertion/deletion type; the two ACE alleles differ in size because of the insertion of a 287-bp DNA sequence in intron 16 of the ACE gene[74]. The ACE polymorphism was initially detected by restriction fragment length polymorphism (RFLP) analysis and Southern hybridization with a human ACE cDNA probe[94]. Subsequent studies of ACE polymorphism and disease associations used polymerase chain reaction (PCR) for genomic DNA amplification. The first PCR-based detection of the I/D ACE polymorphism (Fig-12) was reported by Rigat et al[74] who used a set of primers flanking the insertion sequence; the generated amplicons corresponding to the I and D alleles differ in size by the length of insertion sequence (ie, 287 bp) and allow discrimination between the three genotypes: II, ID, and DD.

Parving *et al.* observed that the deletion allele of the ACE polymorphism reduces the long-term beneficial effect of ACE inhibition on the progression of overt diabetic nephropathy in patients with diabetes[95,96]. In contrast, long-term treatment with the angiotensin II-receptor blocker losartan induced a similar rate of decline in glomerular filtration rate (GFR) .

Based on a large, double-blind, randomized study comparing the renoprotective effects of losartan *versus* placebo on top of conventional blood pressure-lowering drugs in proteinuric type 2 diabetic patients, we demonstrated that the deletion allele of the ACE gene had a harmful impact on the composite endpoint of doubling of baseline serum creatinine concentration, ESRD, or death. The D allele carriers had the worst renal prognosis in all ethnic groups. It should be stressed that demographic data, clinical history, and laboratory variables at baseline by genotype were similar between the losartan and placebo groups with the exception of proteinuria in the II genotype group. The beneficial effects of losartan were greatest in the ACE/DD group and intermediate in the ID group for nearly all endpoints, a trend suggesting a quantitative interaction between losartan treatment and ACE/ID genotype on progression of renal disease. In agreement with a previous analysis of the RENAAL study, losartan exhibited renoprotection in all ethnic groups[97]. The treatment-ACE-genotype interaction was significant for the risk reduction of the ESRD endpoint. The major novel clinical importance of the above study is that those patients who have the greatest need for renoprotective treatment have the best effect of losartan (DD and ID), whereas those patients with a better renal prognosis (II) also derived renal benefit. The finding of similar values

for serum creatinine and albumin/creatinine ratio at baseline in the three genotype groups may well be due to previous renoprotective treatment, which according to the present analysis mitigated differences observed in patients receiving RAAS blockade.

**Effect of Genotype on Enzymatic Levels:**

The ACE DD genotype is associated with increased circulating ACE levels, which are generally two times as high as those found for II genotypes; ID heterozygotes are associated with intermediate ACE levels[98](Fig-11). This relationship of D allele dose and enzymatic levels, originally reported by Rigat et al, was repeatedly confirmed by other studies, for both circulating and cellular ACE[18,99-101]. Increased conversion of angiotensin I to angiotensin II, secondary to the higher ACE concentrations, has been suggested to be a mechanism underlying differences in cardiovascular and renal function/prognosis in subjects with the DDgenotype compared with the II genotype[102,103]. However, because the ACE I/D polymorphism is intronic, the mechanism of ACE overexpression in subjects with DD genotype is unclear. It is thought to be in linkage disequilibrium with a functional mutation in the gene[19].

**Figure-11**

**EFFECT OF ACE GENE POLYMORPHISM**

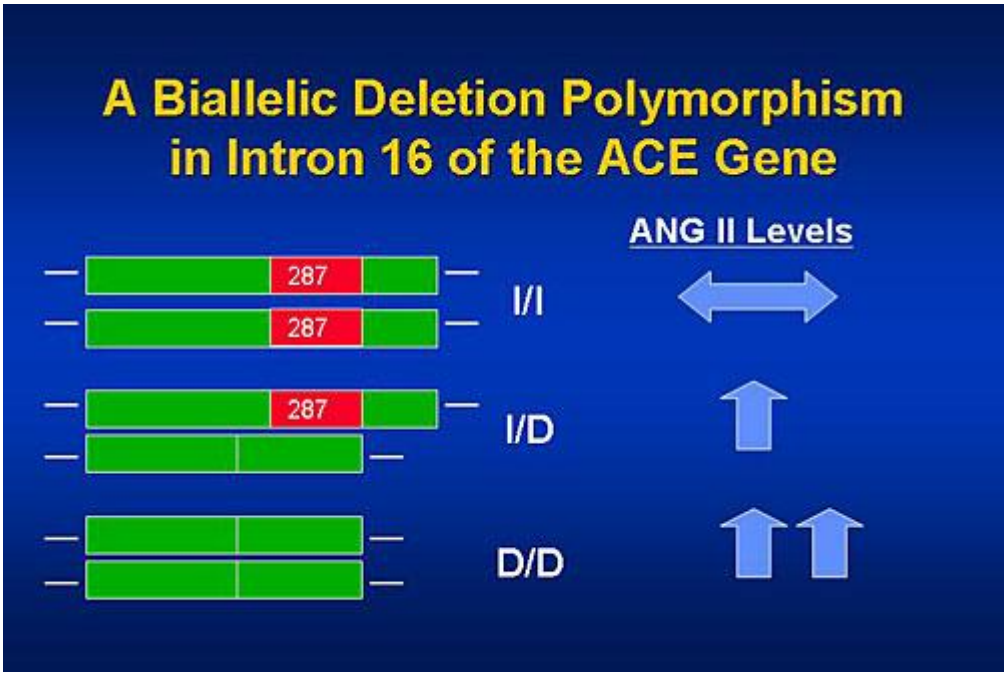



**Figure - 12**

**ACE GENE SHOWING INTRON 16 THE SITE OF INSERTION DELETION POLYMORPHISM**

**Ang II, superoxide anions, and Inflammation:**

Reactive oxygen species (ROS), generated by the membrane-bound NADPH-oxidase system[104] (Fig-13) and stimulated by Ang II via its AT1-receptor, seem to be a pivotal step in glomeulosclerosis; it is possible that this relationship is the result of tight linkage to another locus involved in the regulation of ACE gene expression[105]. DD genotype associated with increased ACE activity results in increased Ang II production and thereby it contributes to glomerulosclerosis. Griendling et al. first demonstrated that Ang II stimulates the generation of ROS in vascular cells and macrophages, which are known activators for cytoplasmic signaling cascades such as nuclear factor-kB (NF-kB), mitogen-activated protein (MAP) kinases, or the Janus tyrosine kinases (JAK)/signal transducers and activators of transcription (STAT) cascade[106,107]. Together, these mechanisms may enhance oxidative stress within the vascular wall and lead to the activation of redox-sensitive genes, such as those for proinflammatory cytokines[108]. These observations suggest that Ang II may, via redox-sensitive mechanisms, activate IL-6 synthesis and release. Moreover, recent observations indicate that proinflammatory eicosanoids, such as leukotriene B4 or thromboxanes, are involved in AT1-receptor-dependent NADPH-

**Figure-13**

**ANGIOTENSIN II IN FREE RADICAL PRODUCTION AND INFLAMMATION**

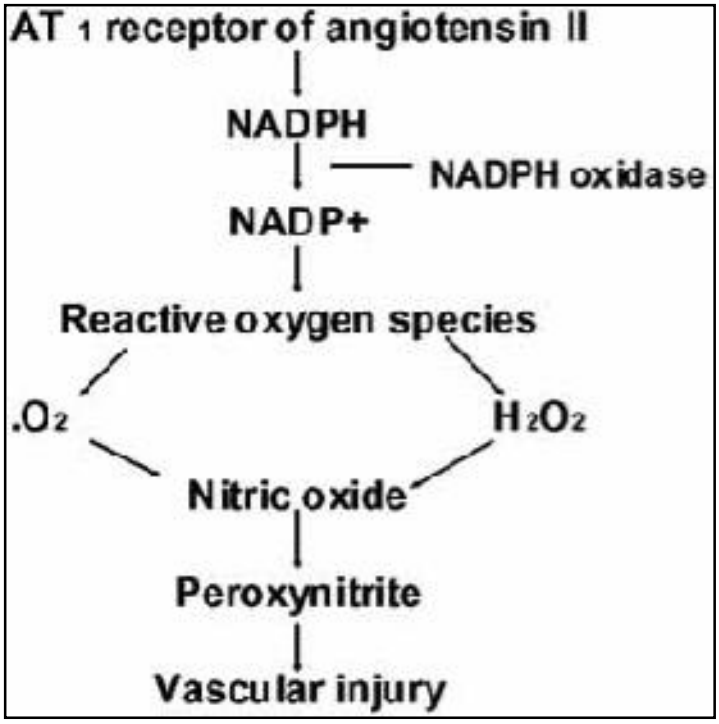



**Table 1. $AT_1$ Receptor and Inflammation**

| | |
|---|---|
| ↑VCAM-1 | ↑TNF-α |
| ↑ICAM-1 | ↑IL-6 |
| ↑E- selection | ↑MMP-2, MMP-9 |
| ↑MCP 1 | ↑VEGF |
| ↑NF-κB | ↑EPC |

oxidase activation. This latter pathway does not only link inflammation with the RAS but also plays a critical role based on its vasoconstrictive and mitogenic potencies.

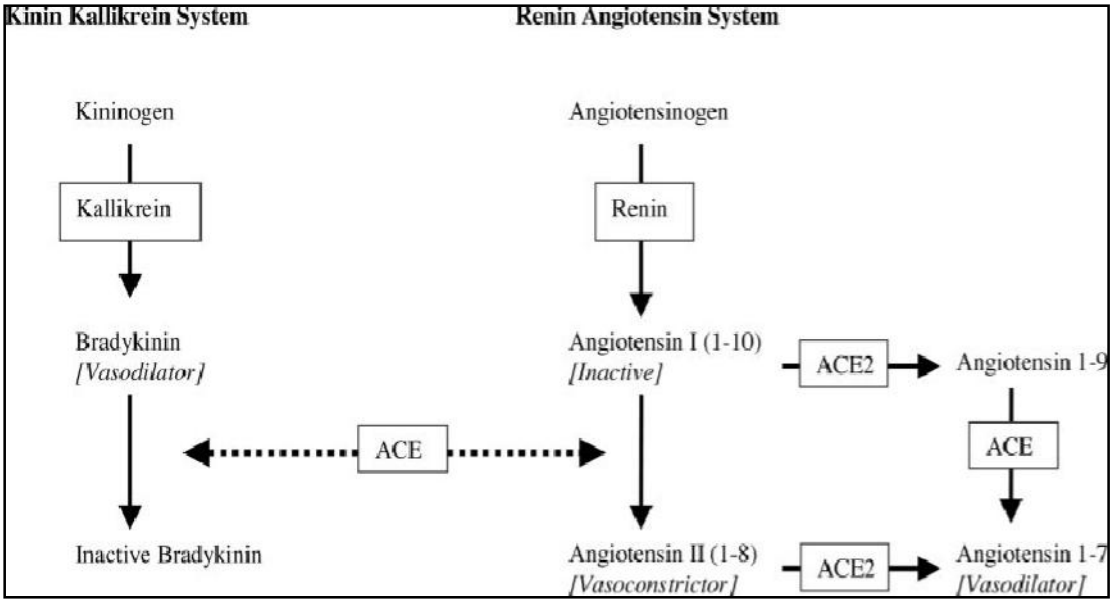
**Other diseases associated with ACE gene polymorphisms** :

**ACE polymorphism and Coronary artery disease**:

$AGTR_1$ is the principal receptor mediating Ang II cardiac and circulatory effects. Cardiac effects include direct inotropic activity resulting in increased myocardial contraction, as well as cell growth and proliferation, resulting in cardiac remodelling, hypertrophy, and ventricular dilatation[93]. $AGTR_2$ appears to be the dominant receptor in both atrial and ventricular myocardium. Ang II can also be generated in the tissues, including myocardium, by pathways other than RAS; non-RAS pathways involve nonspecific carboxypeptidases and chymotrypsin-like proteinases. An example of one of these is chymase (serine-proteinase), which catalyzes an efficient Ang II generation at tissue levels. Production of Ang II by these non-RAS alternative pathways is not inhibited by therapy with ACE inhibitors. The chymase pathway has been demonstrated in various cell types, including myocardium, endothelial cells, and mast cells[109]. Chymase levels have

**Figure-14**

**THE RAS AND KININ SYSTEM**



been found higher in the ventricles than in the atria, and ventricle levels do not appear to change significantly in heart failure[109.] Deletion polymorphism in the for angiotensin converting enzyme is a potent risk factor for myocardial infarction[100].

The interaction between ROS, inflammatory cells, and the RAS seemed to be important not only for the development of acute coronary syndrome, but also for the progression of atherosclerosis . With regard to the development of atherosclerotic lesions, evidence from other animal models, including rodents and primates, shows that ACE inhibition may reduce the extent of vascular lesions[110-112].

Additional mechanisms by which the RAS via Ang II may enhance the development of atherosclerosis involve the activation of thrombosis pathways via PAI-1 [113,114] or the stimulation of pro-inflammatory cytokines. Diet et al.[115] first demonstrated that Ang II–forming protease ACE is expressed in human atherosclerotic plaques. The authors demonstrated that in early- and intermediate-stage atherosclerotic lesions, ACE was predominantly expressed in lipid-laden macrophages (similarly to pro-inflammatory cytokines) whereas in advanced lesions ACE was predominantly localized throughout the

plaques microvasculature[112]. Potter et al. further demonstrated that lipid-laden macrophages contain Ang II in a primate model of atherosclerosis.

In humans, at least two major enzymes, ACE and chymase, are involved in the conversion of Ang I to Ang II and may contribute to Ang II formation in coronary arteries. Further investigations demonstrated that in normal and atheromatous coronary artery segments of patients dying of noncardiovascular diseases that only ACE, but not chymase, was co localized with Ang II in the intima of stable atherosclerotic plaques. These findings suggest that ACE is apparently the primary source of Ang II in atherosclerotic human coronary arteries. Interestingly, Hoshida et al. demonstrated that tissue ACE activity is selectively upregulated in patients with ACS but serum-ACE activity is not. These observations suggest that tissue-ACE activity may represent an important regulator of Ang II formation at the atherosclerotic lesion.

**ACE Polymorphism and Venous Thrombosis**

The DD genotype was found as a potent risk factor for thrombosis in patients undergoing total hip arthroplasty[116]. In a case-controlled study, Philipp et al investigated the association of ACE polymorphism, with postoperative venous thrombosis. The plasma ACE levels in this

study showed the same pattern previously reported by others, with the highest values in DD patients, intermediate values in ID heterozygotes, and the lowest values in II patients[116]. Another study that showed an association between the DD genotype and an increased risk of venous thrombosis was reported by Dilley et al[117] for an African-American population, with a threefold relative risk in men but not in women.

## ACE Polymorphism and Coronary Restenosis after Stent Implantation

Because RAS has been implicated in the development of neointimal hyperplasia,[118] ACE activity is a crucial step for the RAS pathway, and the resulting increased generation of Ang II is a potent growth factor for smooth muscle cells[119], the hypothesis has been advanced that genetic factors affecting RAS and particularly ACE gene expression may be important in pathogenesis of coronary restenosis after stenting. Indirect experimental evidence was obtained by demonstrating that ACE inhibitors block neointimal thickening after arterial balloon denudation in rats, guinea pigs, and rabbits[120,121]. The clinical relation between restenosis after coronary stenting and ACE polymorphism was investigated by Amant et al[122] ; the association of the number of D alleles and poststent restenosis was independent of other risk factors[122].

The increased ACE activity due to the presence of the D allele, mainly in the homozygous state, may account for the higher degree of coronary neointimal thickening found in these patients[122].

**ACE Polymorphism and Hypertension**

The association between ACE polymorphism and essential hypertension is controversial. A significant association of the ACE gene D allele with essential hypertension was documented in the African-American (Duru *et al*., 1994), Chinese (Chiang *et al*., 1996), and Japanese populations(Morise *et al*., 1994; Nakano *et al*., 1998). On the other hand,the I allele was associated with high blood pressure in an Australian population with strong evidence of familial hypertension (Zee *et al.,* 1992). It has been suggested that the population heterogeneity in the association of ACE I/D polymorphism with essential hypertension may be due to significant variations of population backgrounds (Barley *et al*.,1994). Response to ACE inhibitors in hypertensive patients appears to be determined at least in part by the ACE genotype in the study of Ohmichi et al[123].

**ACE polymorphism and breast cancer:**

Evidence from animal models has suggested that angiotensin II stimulates neovascularisation by promoting arteriolar smooth muscle cell proliferation, and this has led to increased interest in the role that angiotensin II may play a role in promoting angiogenesis in neoplastic growth. In addition, angiotensin II may act as a mitotic factor by inducing or regulating gene expression in cell cycle progression, and angiotensin II receptor blockade effectively reduced transforming growth factor ß1-dependent tumor progression *in vivo* . Captopril, a prototype ACE inhibitor, has been shown to inhibit proliferation in a variety of cell types, including human breast cancer cells[123-126,] and to reduce tumour growth in experimental models of cancer[127].

Ace polymorphism was also studied in other diseases like cardiomyopathy, stroke, Alzheimer's disease, Systemic Sclerosis.

# AIM OF THE STUDY

# AIMS AND OBJECTIVES

Diabetic nephropathy is a common cause of chronic kidney disease especially in developing countries. It is a major contributor to mortality in patients with type 2 diabetes mellitus. Genetic factors appear to play an important role in susceptibility to diabetic nephropathy because it affects only one third of subjects. The knowledge of genetic factors of diabetic nephropathy may help in explaining the molecular basis of this disorder and in designing prevention and treatment methods. Literature evidences point to the role of ACE gene I/D polymorphism in the causation of glomerulosclerosis .Even though ACE gene polymorphism is located in intron 16 of ACE gene, it is thought to be in linkage disequilibrium with another functional mutation in the gene. The differences in the degree of linkage disequilibrium between this quantitative trait locus and I/D polymorphism are cited as a reason for the differences in the associations of the polymorphisms with diabetic nephropathy in different populations.

The aim of the study is

1 To find out distribution of ACE gene polymorphism among healthy controls , type 2 diabetic patients with and without nephropathy.
2 To assess serum ACE activity among study groups and correlate with the genotype .

# MATERIALS AND METHODS

# MATERIALS AND METHODS

This is a case-control study and was conducted after obtaining ethical committee clearance. The study was carried out during the period April 2011- October 2011 at Madras medical college and Rajiv Gandhi government general hospital.

**Study population:**

**CASES:**

60 unrelated type 2 diabetic patients with duration of diabetes more than five years attending diabetic outpatient department of our hospital were included in the study after obtaining consent and were categorised into

**Group 1A:**

30 (14 males, 16 females) type 2 diabetics with nephropathy and

**Group 1B:**

30 (11 males, 19 females) type 2 diabetics without nephropathy based on the early morning urine albumin creatinine ratio (ACR). Urine ACR was estimated with three times in a six month period and patients who tested positive on two out of three measurements were included in the diabetic nephropathy group.

Patients with Urine Albumin Creatinine ratio of

$\geq$ 23 mg/g of creatinine for males and

$\geq$ 32 mg/g of creatinine for females[128]

were included in the nephropathy group.

**Exclusion criteria:**

- Known hypertensives on treatment
- Those who had BP > 140/90 mmHg
- Known heart disease patients
- Those who are on ACE inhibitors were excluded from the study.

**CONTROLS:**

30 age and sex (15 males and 15 females) matched healthy individuals attending master health check- up were selected as controls.

**Sample collection:**

**Blood samples:**

Blood was collected after an overnight fast of 8-12 hrs. About 5mL of blood was drawn from the cubital vein of the subjects. 2 mL was

transferred into EDTA tubes and 3 mL into plain tubes. The blood samples were analysed on the same day within an hour of collection.

DNA extraction was done on the same day and extracted DNA stored at -20°C. Also 1mL of serum transferred into 1.5mL eppendorf was stored at -20°C for measuring ACE activity.

**Urine samples:**

Early morning urine samples were collected in sterile plastic containers and Albumin Creatinine ratio was estimated.

**METHODS:**

**BUFFY COAT SEPARATION**

Buffy coat was separated by centrifugation of EDTA tubes at 2000 revolutions per minute for 20 minutes. Buffy coat was transferred to 2mL eppendorf and was used for DNA extraction.

**DNA EXTRACTION BY MODIFIED HIGH SALT METHOD[129]**

**RBC Lysis:**

- 400µL of buffy coat in a 2mL eppendorf is mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion until red cells are lysed for about 10 minutes

- The cells are centrifuged at 4000rpm for 10minutes.
- The white cell pellet is washed with 800µL of 0.17M ammonium chloride solution. The procedure is repeated till a clear white cell pellet is obtained.

**WBC Lysis**

- To the pellet 500 µL of TKM I solution is added. It is centrifuged at 10,000rpm for 10minutes.

**Nuclear Lysis**

- Discard the supernatant. To the pellet add 500 µL of TKM II solution. To that add 300 µL of 6M Nacl and 50 µL of 10% SDS.
- Mix well (vortex), Centrifuge at 10,000 rpm for 10 minutes.
- Save the supernatant. Transfer it to 1.5mL eppendorf.

**DNA Precipitation**

- To the supernatant double the volume of 100% ethanol is added.
- The sample is stored at -20◦C for 1 hour.
- Then it is centrifuged at 10,000 rpm for 20minutes at 4◦C in a refrigerated centrifuge.

- The supernatant is discarded. To this 500 µL of 70% ethanol is added. The pellet is mixed and centrifuged at 10,000 rpm for 10minutes at 4◦C.
- Supernatant is discarded and the pellet is air dried.

**Storage**

- To the pellet 30 µL of LTE buffer is added and the extracted DNA is stored at -20◦C for future use.

**Identification**

- Extracted DNA was identified by 0.8% agarose gel electrophoresis with a constant voltage of 7V/ cm and comparison with a known molecular weight 1kb DNA ladder.Figure:

**Concentration of extracted DNA:**

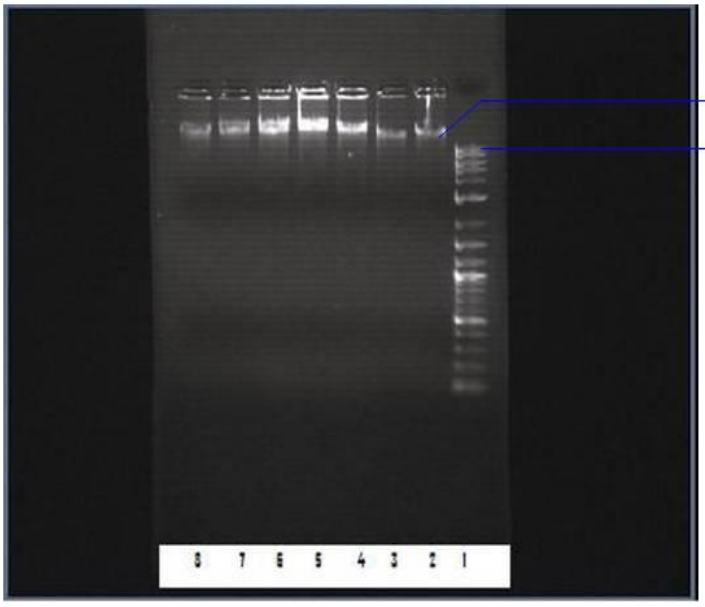
- Concentration of extracted DNA was estimated using UV spectrophotometer at 260 nm.
- Concentration was calculated using the formula :

  1 OD is equivalent to 50 µg/mL.

  Conc. of DNA = absorbance X 50 µg/mL X dilution factor

  = y X 50 X 100 ng/µL
- Purity of extracted DNA was assessed by 260nm/280nm ratio.

# DNA EXTRACTION BY HIGH SALT METHOD




- Extracted DNA (lane 2 to 8) was tested on 1% agarose gel using 1kb ladder (lane 1)
- Ladder shows 10000, 8000, 7000,6000, 5000, 4000, 3000, 2000 1000 kbp fragments

**Estimation of Fasting plasma glucose:**

**Method:** Glucose oxidase peroxidase (GOD/POD), Enzymatic method

**Principle:**

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenolic compound} + \text{4aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Pink coloured complex} + 2H_2O$$

The intensity of pink coloured compound is proportional to glucose concentration and was measured at 505nm.

**Procedure:**

To 1 ml of working reagent, 10 µL of plasma was added and incubated at 37°C for 15 min and absorbance was measured at 505nm.

**Reference range :** Fasting plasma glucose - → 70 - 100 mg/dL

**Estimation of Blood Urea:**

**Method : GLDH method, Enzymatic method**

**Principle:**

The test is performed as a kinetic assay in which the initial rate of the reaction is linear for a limited period of time.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_4 + CO_2$$

$$NH_4 + NADH + H^+ + \text{2-Oxoglutarate} \xrightarrow{\text{GLDH}} \text{Glutamate} + NAD^+$$

The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample.

**Procedure:**

To 1 mL of working reagent 10 µL of sample or standard is added. Absorbance is measured after 30 sec (A1) and 90 sec (A2).

**Calculation:**

$$\frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \text{ X } 50 \text{ (Standard Conc)} = \text{mg/dL urea in the sample}$$

**Reference range:** Normal blood urea = 15 to 40 mg/dL

**Estimation of Serum Creatinine:**

**Method :** Modified Jaffe's Method.

**Principle**:

Creatinine reacts with alkaline picrate to produce an orange-yellow coloured complex (creatinine picrate). The absorbance of orange-yellow colour formed is directly proportional to creatinine concentration and is measured photometrically at 520 nm.

**Procedure:**

To 500 mL of working reagent 50 µL of serum sample or standard is added and initial absorbance (A1) is measured at 20 sec and final absorbance (A2) is measured at 80 sec after mixing.

**Calculation:**

$\Delta A = A_2 - A_2$

$$\text{Creatinine (mg/dL)} = \frac{\Delta A \text{ of Test}}{\Delta A \text{ of Standard}} \times \text{Concentration of Standard (2 mg/dl)}$$

**Normal range :** 0.6-1.2 mg/dL

**Estimation of Urine Creatinine:**

Estimation of urine creatinine is done in the same method( modified Jaffe's) as serum creatinine measurement except for diluting

the urine sample ten times and multiplying the obtained result with dilution factor.

Normal Urine creatinine excretion is 1-2 g/day

**Estimation of Urine Microalbumin :**

**Method:** Immunoturbidimetry method.

**Principle :**

Latex particles coated with specific antibodies anti-human albumin are agglutinated when mixed with samples containing microalbumin. The agglutination causes an absorbance change dependent upon the µALB contents of the patient sample and quantified by comparison with a calibrator of known µALB concentration.

**Procedure :**

To 1 mL of the working reagent 7µL of calibrator or sample is added and absorbance measured immediately($A_1$) and after 2 min ($A_2$) of sample addition. The temperature of the reagent and the reaction mixture are maintained at 37°C.

**Calculations:**

$$\frac{(A_2 - A_1)_{sample}}{(A_2 - A_1)_{calibrator}} \text{ X Calibrator concentration = mg/L albumin}$$

Calibrator of concentration 64 mg /L was used.

Normal : 30 mg/24 hrs urine specimen

20 mg/L in early morning sample

**Estimation of Urine Albumin Creatinine Ratio (ACR) :**

Urine microalbumin per gram of Creatinine excreted was calculated for each patient sample.

ACR $\geq$ 23 mg/g of creatinine for males and

$\geq$ 32 mg/g of creatinine for females is considered as nephropathy range.

**Estimation of Serum ACE activity :**

**Method:** Serum ACE activity was measured spectrophotometrically at 340 nm.

**Principle :** The following reaction is catalysed by ACE:

FAPGG $\longrightarrow$ FAP + Glycylglycine

FAPGG ( N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine) is hydrolysed to furylacryloylphenylalanine (FAP) and glycyl glycine.

Hydrolysis of FAPGG results in a decrease in absorbance at 340 nm. The ACE activity in the sample is determined by comparing the sample reaction rate to that obtained with the ACE calibrator.

**Procedure:**

The temperature of the reaction mixture was maintained at 37°C.

To 1 ml of ACE reagent 100µL of calibrator or serum sample added, mixed well.

Constant temperature maintained using a water bath and after 5 min absorbance recorded which is the initial absorbance. Exactly after 5 min again absorbance was recorded. This is the final absorbance.

**Calculation**:

ΔA per 5 min (Test) = Initial A Test – Final A Test

ΔA per 5 min (Calibrator) = Initial A Calibrator – Final A Calibrator

$$\text{ACE (U/L)} = \frac{\Delta A/5 \text{ min of Test}}{\Delta A/5 \text{ min of Calibrator}} \times \text{Activity of calibrator}$$

Absorbance values were calculated using an ACE Calibrator with an activity of 50µL at 37°C.

Normal range : 8 – 52 U/L at 37°C.

## POLYMERASE CHAIN REACTION

ACE gene was amplified using,

- Forward primer – 5'-CTGGAGACCACTCCCATCCTTTCT–3' and
- Reverse primer – 5'-GATGTGGCCATCAATTCGTCAGAT – 3'

### Primer Reconstitution

Primers are supplied in lyophilized form.

Autoclaved distilled water is used to prepare 100 × concentrations i.e. 10times the molecular weight of primer is the volume of water required to prepare 100 × concentrations which is 100µmolar solution.

- From this stock solution 10 × concentration is prepared as the working solution for PCR.

### MASTER MIX:

- Genei Red Dye master mix in the following composition was used.
- Master Mix consists of a unique inert red dye in addition to basic components necessary for PCR.
  - Reaction buffer consisted of Tris Hcl -10mM at pH 8.3
    KCl - 50mM
  - MgCl2 - 1.5mM acts as catalyst.
  - dNTP's were used in a concentration of 2.5mM each.
  - Taq polymerase in a concentration of 1.5 U.

- Primers were used in a concentration of 5 pmol and DNA was used in a concentration of 200ng.

- PCR was carried out in a reaction volume of 12.5 µL with the following components;
  - PCR master mix – 6.5 µL
  - Forward primer – 0.5 µL
  - Reverse prime – 0.5 µL
  - DNA – 1.0µL
  - Distilled water – 4.0 µL
  - Total – 12.5 µL

- Amplification was carried out in an Mc Genei thermal cycler with the following cycling conditions.
  - Initial denaturation – $94^0$ C -5min
    - 30 cycles of
    - Denaturation – $94^0$C – 1 min
    - Annealing - $58^0$C – 1min
    - Extension -$72^0$C – 1min
  - Final extension at $72^0$C - 10 min.

- Amplified products 490 bp PCR for I allele and 190bp PCR product for D allele was identified by agarose gel electrophoresis by comparison with a known 100bp DNA ladder. Thus, each DNA sample revealed one of three possible patterns after electrophoresis: a 490 bp band (II genotype), a 190 bp band (DD genotype), or both 490 and 190 bp bands (I/D genotype) Figure .

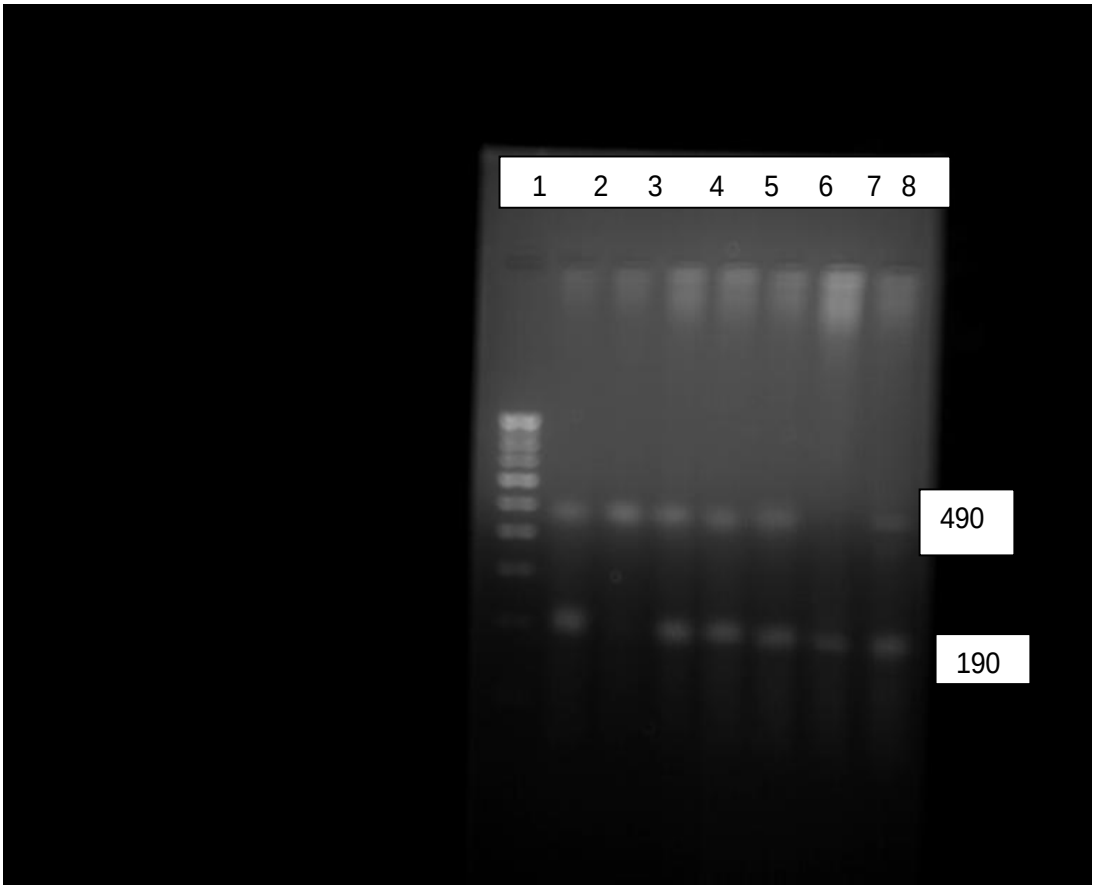
**AGAROSE GEL ELECTROPHORESIS**

- PCR product is run on agarose gel in a 50 mL agarose cast as follows: 1g of agarose is weighed and dissolved in 50mL of TAE buffer with a pH of 8.0.

- It is microwaved for 60 secs, cooled and 2.5 µL of ethidium bromide (10mg/mL) is added. It is poured into a cast and allowed to solidify for 15 min before it is kept in the electrophoresis tank.

- 8 µL of PCR product is loaded onto wells and 4 µL of 100bp DNA ladder is loaded onto single well as a marker. It is electrophoresed at 8V/cm for 45min and visualized under UV illumination.

**AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS**




**Agarose gel electrophoresis of PCR products. Deleted allele has 190bp product, Inserted allele has 490 bp product. Lane 1 shows ladder (100,200,300,400,500,600,700,800,900,1000bp), lanes 2,4,5,6,8 shows both the products indicating ID genotype, lanes 7 shows only 190bp product indicating DD genotype, lane 3 shows only 490 bp product indicating II genotype.**

# STATISTICAL ANALYSIS

# STATISTICAL ANALYSIS

1. Waist-Hip ratio, Fasting plasma glucose, Blood urea, Serum creatinine, Urine microalbumin, Urine Creatinine and Urine Albumin Creatinine ratio were compared between three study groups by ANOVA.

2. ACE Genotype frequency distribution between cases and controls were compared with ANOVA.

3. Odds ratio was calculated for ACE genotype distribution in the study population.

4. Allele frequencies were calculated by allele counting.

5. ACEactivity was compared between the study groups by ANOVA.

6. ACE activity for the ACE genotypes were compared by ANOVA.

Statistical analysis was done using SPSS software.

# RESULTS

## CASES - Group 1A – DIABETICS NEPHROPATHY

| Sl. No. | Age | Sex | Duration of diabetes | Waist circum-ference (cm) | Hip circum-ference (cm) | Waist Hip ratio | Fasting Plasma Glucose (mg/dL) | Blood Urea (mg/dL) | S.Creatinine (mg/dL) | U.Creatinine (g/dL) | U.MicroAlbumin (mg/L) | Albumin creatinine ratio (mg/g of creatinine) | ACE poly-morphism | ACE activity (U/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 65 | M | 6 | 80.5 | 90.5 | 0.89 | 162 | 18 | 0.9 | 0.8 | 149 | 186 | DD | 73 |
| 2 | 50 | M | 6 | 81.5 | 93.5 | 0.87 | 189 | 20 | 1 | 1.1 | 73 | 66 | ID | 52 |
| 3 | 55 | M | 6 | 77.5 | 86.5 | 0.9 | 104 | 25 | 1.1 | 1 | 76 | 76 | ID | 61 |
| 4 | 60 | M | 6 | 94.5 | 100.5 | 0.94 | 289 | 32 | 0.7 | 0.9 | 83 | 92 | DD | 115 |
| 5 | 52 | M | 6 | 70 | 88 | 0.8 | 129 | 20 | 1.1 | 0.9 | 39 | 43 | ID | 55 |
| 6 | 57 | F | 6 | 77 | 96 | 0.8 | 140 | 26 | 1.1 | 0.8 | 40 | 50 | DD | 81 |
| 7 | 50 | M | 6 | 78 | 92 | 0.85 | 308 | 26 | 0.9 | 1 | 39 | 39 | II | 41 |
| 8 | 53 | F | 7 | 92.5 | 95.5 | 0.97 | 85 | 40 | 0.8 | 1 | 100 | 100 | DD | 88 |
| 9 | 57 | M | 7 | 96.4 | 105 | 0.92 | 170 | 26 | 1 | 1 | 54 | 54 | ID | 57 |
| 10 | 59 | F | 8 | 101.5 | 117 | 0.87 | 157 | 52 | 1 | 1.3 | 109 | 84 | DD | 79 |
| 11 | 55 | F | 10 | 84 | 98 | 0.86 | 152 | 22 | 1.2 | 0.9 | 77 | 86 | DD | 97 |
| 12 | 65 | F | 10 | 83.5 | 95.5 | 0.87 | 127 | 29 | 0.9 | 0.9 | 43 | 48 | ID | 53 |
| 8 | 58 | F | 10 | 93.5 | 102.5 | 0.91 | 259 | 21 | 1 | 1 | 52 | 52 | DD | 81 |
| 14 | 60 | F | 10 | 89.5 | 98.5 | 0.91 | 164 | 20 | 0.8 | 1.9 | 86 | 43 | II | 16 |
| 15 | 60 | F | 10 | 86.5 | 92.5 | 0.94 | 270 | 37 | 0.8 | 0.9 | 85 | 94 | DD | 83 |
| 16 | 61 | F | 10 | 80.5 | 92.5 | 0.87 | 87 | 31 | 1.1 | 0.9 | 78 | 87 | DD | 67 |
| 17 | 50 | F | 10 | 81.5 | 92.5 | 0.88 | 286 | 52 | 0.9 | 0.8 | 34 | 42 | DD | 86 |
| 18 | 56 | M | 10 | 82 | 93 | 0.88 | 127 | 25 | 0.9 | 1.4 | 115 | 82 | ID | 61 |
| 19 | 45 | F | 10 | 86 | 98 | 0.88 | 214 | 28 | 1.1 | 1 | 125 | 125 | DD | 68 |
| 20 | 58 | M | 11 | 91.5 | 96.5 | 0.95 | 235 | 23 | 1 | 1.1 | 43 | 38 | II | 23 |
| 21 | 47 | F | 11 | 85 | 107 | 0.79 | 148 | 30 | 1 | 0.8 | 57 | 71 | ID | 56 |
| 22 | 53 | M | 12 | 96 | 100 | 0.96 | 187 | 25 | 0.9 | 1.1 | 132 | 120 | II | 34 |
| 23 | 63 | F | 12 | 92.5 | 102.5 | 0.9 | 138 | 24 | 1.1 | 1 | 54 | 54 | DD | 112 |
| 24 | 42 | F | 12 | 80 | 93 | 0.86 | 131 | 28 | 0.9 | 1.1 | 79 | 72 | DD | 72 |
| 25 | 62 | M | 13 | 83 | 92 | 0.9 | 88 | 22 | 1 | 0.8 | 45 | 56 | ID | 54 |
| 26 | 63 | M | 15 | 76.5 | 87.5 | 0.87 | 131 | 30 | 1.1 | 0.9 | 59 | 66 | DD | 76 |
| 27 | 65 | F | 15 | 78.5 | 98.5 | 0.8 | 204 | 26 | 0.9 | 1.2 | 108 | 90 | ID | 59 |
| 28 | 64 | M | 15 | 65.5 | 77.5 | 0.85 | 264 | 27 | 1.1 | 0.9 | 118 | 131 | DD | 70 |
| 29 | 61 | M | 15 | 102.5 | 109.5 | 0.94 | 88 | 38 | 1 | 0.8 | 41 | 51 | DD | 84 |
| 30 | 45 | F | 7 | 83 | 105 | 0.79 | 192 | 31 | 0.9 | 1 | 60 | 60 | DD | 68 |

## CASES - Group 1B – DIABETICS WITHOUT NEPHROPATHY

| Sl. No. | Age | Sex | Duration of diabetes | Waist circum-ference (cm) | Hip circum-ference (cm) | Waist Hip ratio | Fasting Plasma Glucose (mg/dL) | Blood Urea (mg/dL) | S.Creatinine (mg/dL) | U.Creatinine (g/dL) | U.MicroAlbumin (mg/L) | Albumin creatinine ratio (mg/g of creatinine) | ACE poly-morphism | ACE activity (U/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 53 | F | 6 | 90 | 100 | 0.9 | 135 | 32 | 1 | 1.7 | 13 | 7.6 | ID | 53 |
| 2 | 60 | F | 6 | 91.5 | 112.5 | 0.81 | 110 | 22 | 0.8 | 1.1 | 10 | 9.5 | ID | 50 |
| 3 | 54 | F | 6 | 91.5 | 103.5 | 0.88 | 146 | 20 | 0.7 | 1 | 20 | 20 | ID | 60 |
| 4 | 40 | F | 6 | 95 | 105 | 0.9 | 123 | 22 | 0.8 | 0.9 | 20 | 22 | ID | 57 |
| 5 | 47 | F | 6 | 72 | 95 | 0.76 | 149 | 30 | 0.8 | 1.4 | 17 | 12 | ID | 52 |
| 6 | 57 | M | 7 | 90.5 | 96.5 | 0.94 | 166 | 34 | 1.1 | 1 | 19 | 19 | II | 28 |
| 7 | 55 | F | 7 | 91.5 | 100.5 | 0.91 | 217 | 28 | 1 | 0.8 | 12 | 15 | DD | 61 |
| 8 | 43 | M | 7 | 84.5 | 94.5 | 0.89 | 132 | 37 | 1.1 | 1 | 5 | 5 | II | 18 |
| 9 | 65 | M | 7 | 85.5 | 94.5 | 0.9 | 475 | 30 | 1 | 1.3 | 26 | 20 | DD | 65 |
| 10 | 54 | F | 7 | 92.5 | 101.5 | 0.91 | 130 | 25 | 0.8 | 0.9 | 12 | 13 | II | 32 |
| 11 | 53 | F | 7 | 80 | 96 | 0.83 | 126 | 21 | 1 | 0.8 | 2.4 | 3 | ID | 48 |
| 12 | 57 | F | 7 | 82 | 97 | 0.85 | 100 | 33 | 0.9 | 1.4 | 22 | 15 | II | 7 |
| 13 | 47 | M | 7 | 82 | 93 | 0.88 | 142 | 28 | 1 | 1.7 | 10 | 5 | ID | 54 |
| 14 | 48 | M | 8 | 80 | 93 | 0.86 | 184 | 22 | 1 | 1.3 | 18 | 14 | II | 26 |
| 15 | 55 | F | 8 | 76 | 95 | 0.8 | 128 | 24 | 1 | 0.9 | 8.4 | 10 | ID | 64 |
| 16 | 48 | M | 8 | 86 | 97 | 0.89 | 139 | 26 | 1 | 0.9 | 10 | 11 | II | 22 |
| 17 | 60 | F | 9 | 106.5 | 115.5 | 0.92 | 123 | 20 | 0.8 | 0.8 | 16 | 20 | ID | 75 |
| 18 | 60 | F | 9 | 91 | 106.5 | 0.85 | 239 | 19 | 0.8 | 1.2 | 13 | 11 | II | 12 |
| 19 | 49 | M | 9 | 93.5 | 98.5 | 0.95 | 160 | 24 | 1.1 | 1.7 | 17 | 10 | DD | 69 |
| 20 | 64 | M | 10 | 73.5 | 81.5 | 0.9 | 96 | 37 | 1.1 | 0.8 | 9 | 1 | ID | 51 |
| 21 | 40 | F | 10 | 92.5 | 102.5 | 0.9 | 153 | 23 | 0.8 | 0.8 | 7 | 8 | II | 34 |
| 22 | 59 | F | 10 | 83.5 | 103.5 | 0.81 | 76 | 22 | 0.9 | 1.4 | 18 | 24 | ID | 55 |
| 23 | 40 | M | 10 | 93.5 | 94.5 | 0.99 | 125 | 20 | 1.1 | 1.5 | 15 | 10 | II | 21 |
| 24 | 48 | F | 10 | 90.5 | 111.5 | 0.81 | 135 | 26 | 0.8 | 1.1 | 21 | 19 | II | 13 |
| 25 | 55 | F | 10 | 100 | 114 | 0.88 | 136 | 26 | 1 | 1 | 22 | 22 | DD | 65 |
| 26 | 50 | M | 11 | 94.5 | 106.5 | 0.89 | 178 | 32 | 0.9 | 0.6 | 4.3 | 8 | DD | 80 |
| 27 | 56 | F | 11 | 88.5 | 101.5 | 0.87 | 206 | 38 | 0.8 | 0.8 | 14 | 18 | II | 10 |
| 28 | 61 | M | 11 | 80 | 88 | 0.9 | 128 | 22 | 1 | 1 | 17 | 17 | ID | 51 |
| 29 | 45 | F | 15 | 85 | 99.5 | 0.85 | 120 | 40 | 1.1 | 0.9 | 4.8 | 9 | ID | 56 |
| 30 | 60 | F | 15 | 66.5 | 73.5 | 0.9 | 300 | 28 | 1 | 0.8 | 10 | 13 | DD | 70 |

## CONTROLS

| Sl. No. | Age | Sex | Waist circum-ference (cm) | Hip circum-ference (cm) | Waist Hip ratio | Fasting Plasma Glucose (mg/dL) | Blood Urea (mg/dL) | S.Creatinine (mg/dL) | U.Creatinine (g/dL) | U.MicroAlbumin (mg/L) | Albumin creatinine ratio (mg/g of creatinine) | ACE poly-morphism | ACE activity (U/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 46 | M | 78 | 100 | 0.78 | 100 | 24 | 0.9 | 0.9 | 10 | 11 | ID | 48 |
| 2 | 42 | F | 74 | 90 | 0.82 | 82 | 28 | 0.9 | 1 | 4.5 | 4.5 | II | 20 |
| 3 | 43 | M | 100 | 112.5 | 0.89 | 91 | 21 | 1 | 1.1 | 7 | 6 | ID | 52 |
| 4 | 51 | M | 83 | 94 | 0.88 | 60 | 32 | 1 | 1.4 | 18 | 13 | DD | 47 |
| 5 | 55 | M | 80 | 97 | 0.82 | 112 | 20 | 0.8 | 1 | 12 | 12 | ID | 55 |
| 6 | 60 | M | 87 | 91 | 0.96 | 101 | 40 | 1.1 | 1.6 | 16 | 10 | II | 9 |
| 7 | 50 | F | 89 | 104 | 0.86 | 95 | 22 | 0.7 | 0.8 | 10 | 12.5 | ID | 50 |
| 8 | 63 | M | 85 | 91 | 0.93 | 110 | 26 | 0.9 | 1.2 | 19 | 16 | II | 33 |
| 9 | 59 | F | 89 | 102 | 0.87 | 112 | 25 | 0.9 | 1 | 15 | 15 | DD | 71 |
| 10 | 52 | F | 84 | 102 | 0.82 | 84 | 20 | 0.9 | 0.8 | 2 | 2.5 | II | 15 |
| 11 | 53 | F | 92.5 | 103.5 | 0.89 | 96 | 27 | 1 | 0.8 | 9 | 11 | II | 26 |
| 12 | 55 | M | 76 | 87 | 0.87 | 66 | 36 | 0.9 | 1 | 7.4 | 7.4 | ID | 52 |
| 13 | 48 | F | 96 | 115 | 0.83 | 114 | 30 | 0.8 | 0.9 | 3.6 | 4 | ID | 57 |
| 14 | 51 | M | 93 | 106 | 0.88 | 93 | 31 | 0.9 | 1 | 9 | 9 | ID | 55 |
| 15 | 54 | F | 99 | 129 | 0.77 | 85 | 30 | 0.8 | 0.8 | 2.4 | 3 | II | 19 |
| 16 | 47 | F | 90 | 110 | 0.82 | 70 | 20 | 1 | 2 | 16.8 | 8.4 | II | 23 |
| 17 | 55 | M | 63 | 82 | 0.77 | 66 | 28 | 0.8 | 1.5 | 21 | 14 | ID | 55 |
| 18 | 55 | M | 68 | 102 | 0.67 | 80 | 24 | 0.9 | 1 | 6 | 6 | ID | 57 |
| 19 | 51 | F | 86 | 103 | 0.83 | 95 | 29 | 0.8 | 0.9 | 13 | 14 | ID | 50 |
| 20 | 50 | M | 71 | 93 | 0.76 | 78 | 34 | 0.9 | 1.1 | 7 | 6 | II | 34 |
| 21 | 54 | M | 80 | 96 | 0.83 | 88 | 30 | 0.8 | 0.8 | 5 | 6 | II | 22 |
| 22 | 52 | M | 92 | 105 | 0.88 | 106 | 23 | 0.9 | 1.2 | 20 | 16 | ID | 49 |
| 23 | 43 | F | 92 | 111 | 0.83 | 62 | 37 | 1 | 0.7 | 6 | 8.6 | ID | 64 |
| 24 | 57 | F | 78 | 95 | 0.82 | 110 | 24 | 0.8 | 2 | 10 | 5 | II | 30 |
| 25 | 50 | F | 74 | 95 | 0.78 | 82 | 25 | 0.8 | 0.8 | 4 | 5 | ID | 58 |
| 26 | 62 | F | 91 | 115 | 0.79 | 80 | 36 | 1 | 2.2 | 18 | 8 | II | 25 |
| 27 | 61 | M | 95 | 112 | 0.85 | 101 | 26 | 0.9 | 0.8 | 4 | 5 | ID | 53 |
| 28 | 55 | M | 90 | 106 | 0.85 | 79 | 34 | 0.8 | 1.3 | 17 | 13 | II | 11 |
| 29 | 54 | F | 88 | 98 | 0.90 | 91 | 27 | 1 | 0.9 | 13 | 14 | ID | 54 |
| 30 | 56 | F | 91 | 107 | 0.85 | 83 | 33 | 1 | 1 | 3 | 3 | ID | 56 |

# RESULTS

**Table 1:**

Shows Waist-hip ratio, Fasting plasma glucose, Blood urea, Serum creatinine, Urine microalbumin, Urine Creatinine and Urine albumin creatinine ratio were compared between cases and controls by ANOVA.

**Waist-Hip ratio:**

The mean Waist-Hip ratio for Group 1A (diabetics with nephropathy) was 0.88 while that of Group 1B (diabetics without nephropathy) was 0.87 and that of controls was 0.84. Both the study groups (cases) showed slightly increased waist-hip ratio when compared with controls and the difference is statistically significant(P value=0.002).

**Fasting plasma glucose:**

The mean fasting plasma glucose for Group 1A was 174 mg/dL while that of Group 1B was 159 mg/dL and that of controls was 89 mg/dL. The difference between the groups was found to be statistically significant (P value= 0.000).

**TABLE - 1**

## Comparison of parameters in cases and controls

| Variables | Cases | | Controls | P value |
|---|---|---|---|---|
| | **Group1A** **Diabetics with nephropathy** | **Group1B** **Diabetics without nephropathy** | | |
| Waist-Hip circumference | 0.88± 0.050 | 0.87±0.048 | 0.83± 0.057 | **0.002 – S** |
| Fasting plasma glucose mg/dL | 174.17±65.57 | 159.23±74.74 | 89.07±15.48 | **0.000 – S** |
| Blood Urea mg/dL | 28.47±8.32 | 27.03±6.04 | 28.07±5.46 | 0.697 – NS |
| Serum Creatinine mg/dL | 0.973±0.12 | 0.940±0.12 | 0.897±0.09 | **0.032 – S** |
| Urine Microalbumin mg/L | 75.10±31.992 | 13.76±6.03 | 10.29±5.882 | **0.000-S** |
| Urine Creatinine g/L | 1.01±0.22 | 1.08±0.31 | 1.12±0.39 | 0.384-NS |
| Urine Albumin Creatinine ratio mg/g of creatinine | 75.27±33.11 | 13.04±6.1 | 8.96±4.23 | **0.000 – S** |

**Blood urea:**

The mean Blood urea for group 1A was 28 mg/dL while that of group 1B was 27mg/dL and that of controls was 28 mg/dL. The blood urea values are within normal reference range and there was no statistical significance between the groups(p value=0.697).

**Serum creatinine:**

The mean serum creatinine value for group 1A was 0.97 mg/dL while that of group 1B was 0.94 mg/dL and that of controls was 0.90 mg/dL. Even though the difference is statistically significant (p value=0.032) the serum creatinine values were found to be within normal limits in the study groups.

**Urine microalbumin:**

Urine microalbumin – mean value for group 1A was 75mg/L while that for group 1B was 14mg/L and that of controls was 10mg/L . It is found that urine microalbumin levels are elevated in diabetic nephropathy group and the difference was found to be highly significant statistically (P value=0.000).

**Urine creatinine:**

The mean value for group 1A was 1.01 g/L while that of group 1B was 1.08 g/L and that of controls was 1.12 g/L and there was no statistical difference between the groups (p value=0.384).

**Urine Albumin Creatinine ratio:**

Urine Albumin Creatinine ratio – mean value of group 1A was 75mg/g of creatinine while that of group 1B was 13 mg/g of creatinine and that of controls was 9 mg/g of creatinine and statistically the difference was found to be highly significant(p value=0.000). The results show that urine albumin creatinine ratio is highly elevated in diabetic nephropathy group.

**Table 2**

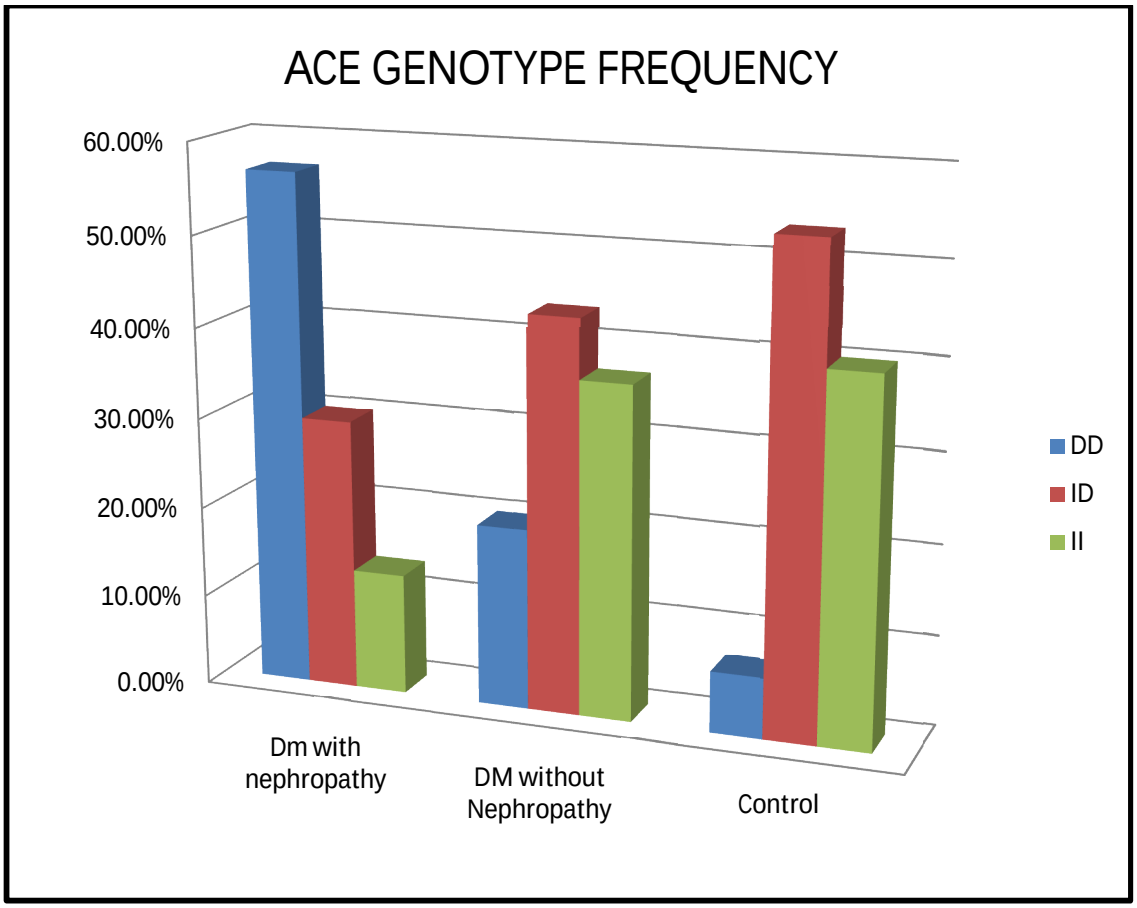
Shows the genotype distribution of ACE gene in Group1A (type 2 diabetic nephropathy), Group 1B (diabetics without nephropathy) and controls.

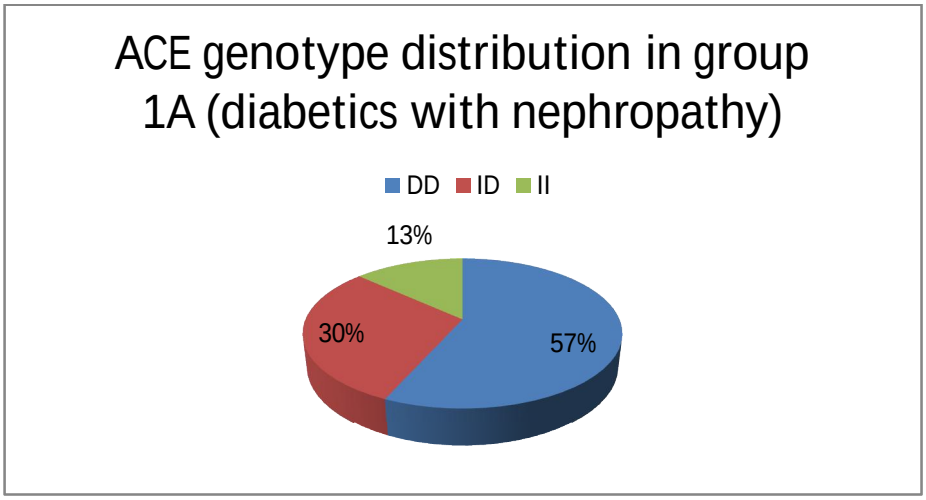
- DD genotype was more frequently distributed among diabetic nephropathy patients 17(56.7%) compared to diabetics without nephropathy 6(20.0%) and controls 2(6.7%). There was a significant

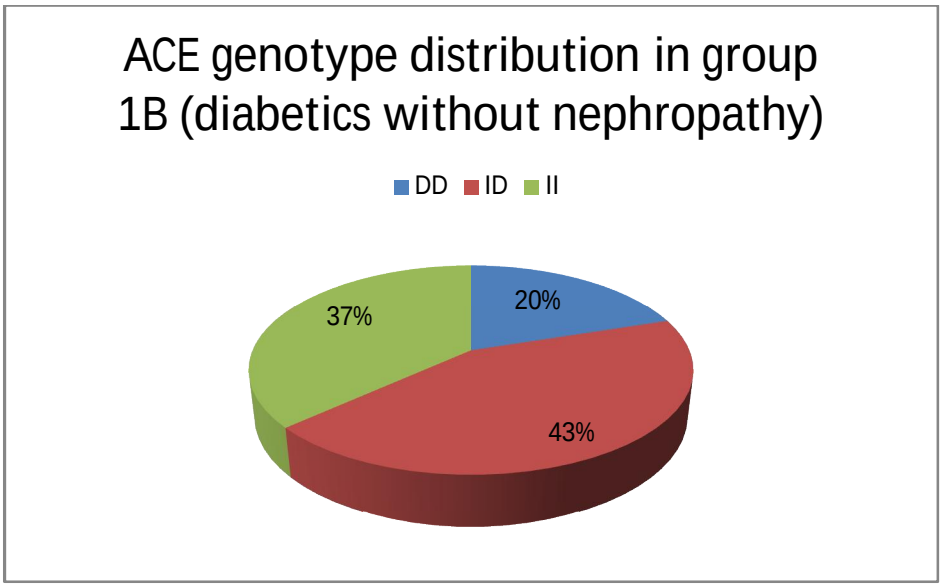
**TABLE – 2**

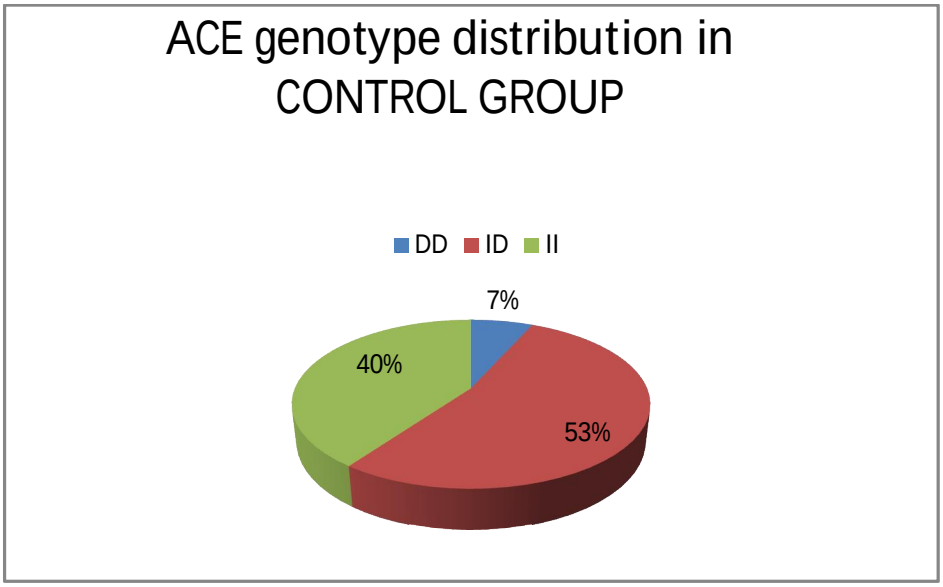
## ACE genotype distribution in study population

| Genotype | Group1A- DM with nephropathy 30 | Group1B- DM without nephropathy 30 | Controls 30 | P value |
|---|---|---|---|---|
| **DD** | 17(56.7%) | 6(20.0%) | 2(6.7%) | **0.000- S** |
| **ID** | 9(30.0%) | 13(43.3%) | 16(53.3%) | |
| **II** | 4(13.3%) | 11(36.7%) | 12( 40.0%) | |




ACE genotype distribution in group 1A (diabetics with nephropathy)
DD
ID
II
13%
30%
57%


ACE genotype distribution in group 1B (diabetics without nephropathy)
DD
ID
II
37%
20%
43%


ACE genotype distribution in CONTROL GROUP
DD
ID
II
7%
40%
53%

difference in the distribution of DD genotype between diabetic nephropathy cases and the other two groups as indicated by the P value (0.000).

- While ID and II genotypes were distributed more in the diabetics without nephropathy and in controls when compared to diabetic nephropathy population.

ACE genotype distribution was in agreement with the Hardy-Weinberg expectations.

**Table 3**

Shows the Odds ratio for ACE genotypes among the study groups.

- The odds ratio for DD genotype between group 1A and group 1B was found as 5.2 and Odds ratio of 3.5 between group 1B and controls and odds ratio of 18 for group 1A and controls.

- The results show that DD genotype has 18 times higher association with diabetic nephropathy compared to controls.

- Other genotypes do not show significant association.

**TABLE – 3**

## ODDS RATIO for ACE genotypes between study groups

| Genotype | Group 1A(Diabetic nepropathy) Vs Group 1B(Diabetics without nephropathy) | Group1B(Diabetics without nephropathy) Vs controls | Group 1A(Diabetic nepropathy) Vs controls |
|---|---|---|---|
| **DD** | 5.2 | 3.5 | 18.30 |
| **ID** | 0.56 | 0.67 | 0.38 |
| **II** | 0.27 | 0.87 | 0.23 |

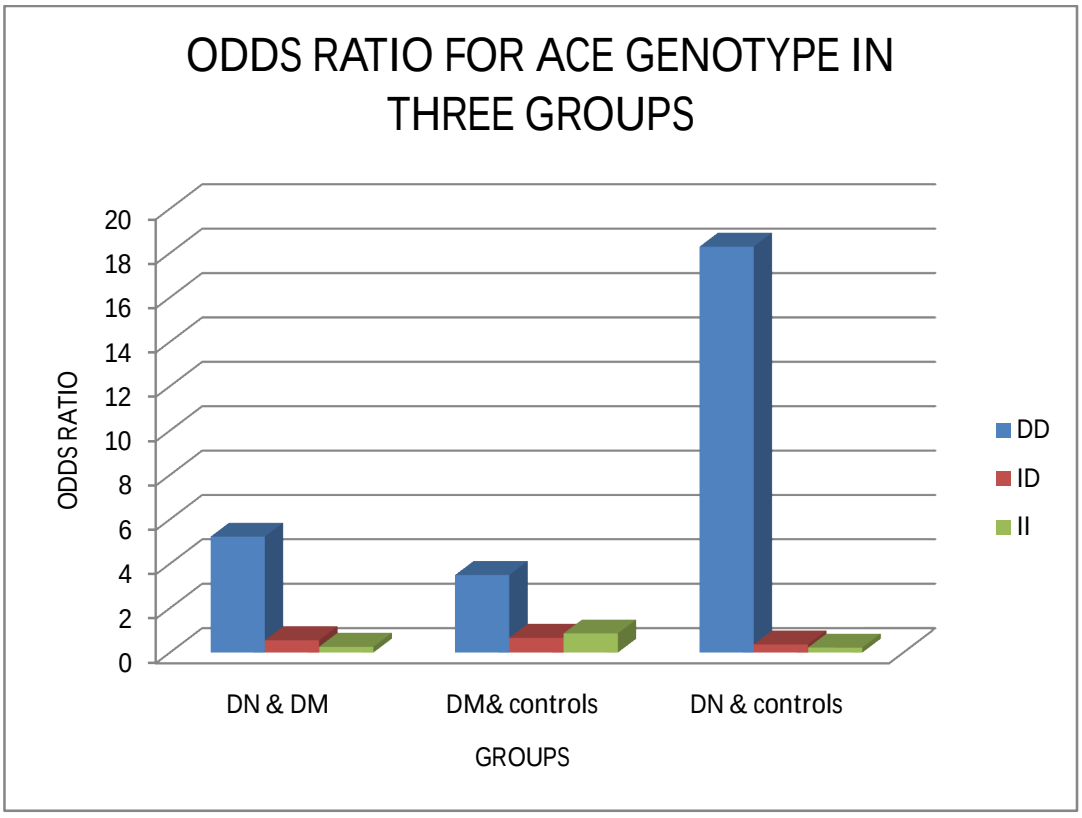


**Table 4**

Shows the Allele distribution of ACE gene between diabetic nephropathy cases and controls.

- It is found that the D+ allele was distributed more commonly in cases . the odds ratio was found to be 4 and p value also significant.

- D+ allele occurs more frequently in cases than controls.

**Table 5**

Shows serum ACE activity among the three groups.

- It is found that serum ACE activity for group 1A(diabetics with nephropathy) was 67 U/L while that of group 1B was 45 U/L and that of controls was 41U/L.

- P value is 0.000, indicates that the difference is statistically significant.

- Our study results indicate that serum ACE activity is elevated in diabetic nephropathy group compared to other groups.

**TABLE-4**

**Distribution of D+ allele in Diabetic nephropathy cases and controls**

| Allele | Group 1A-Diabetic Nephropathy Cases | Controls | P Value |
|---|---|---|---|
| D+ | 26(86.6%) | 18(60%) | 0.020 |
| D- | 4(13.3%) | 12(40%) | Odds ratio= 4 |

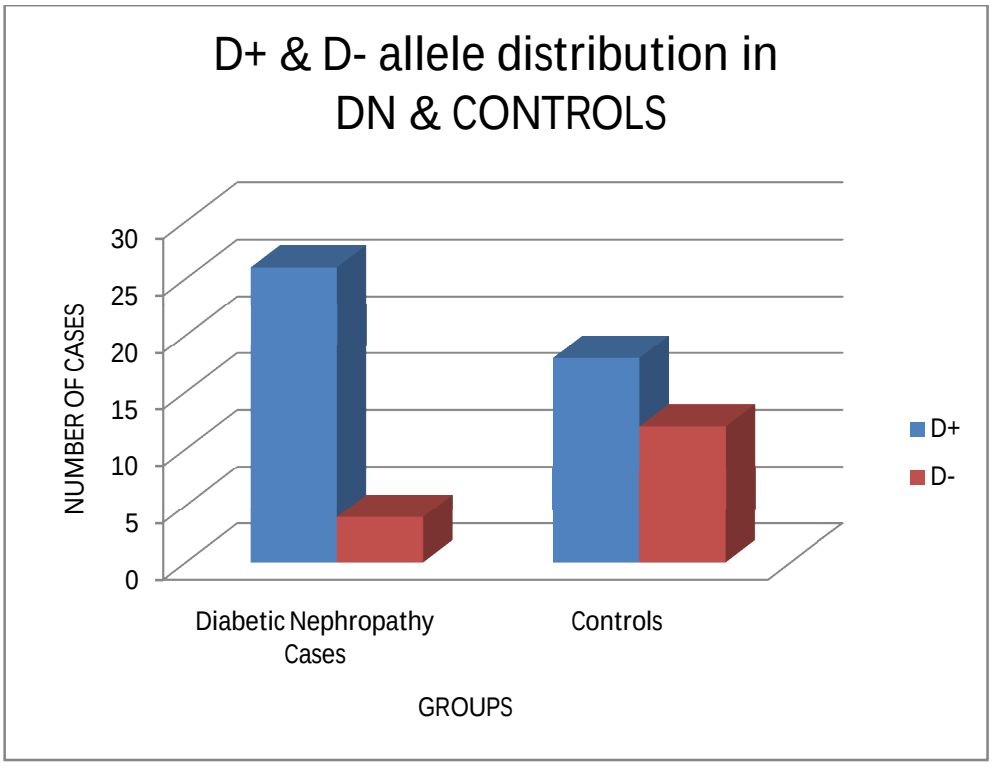
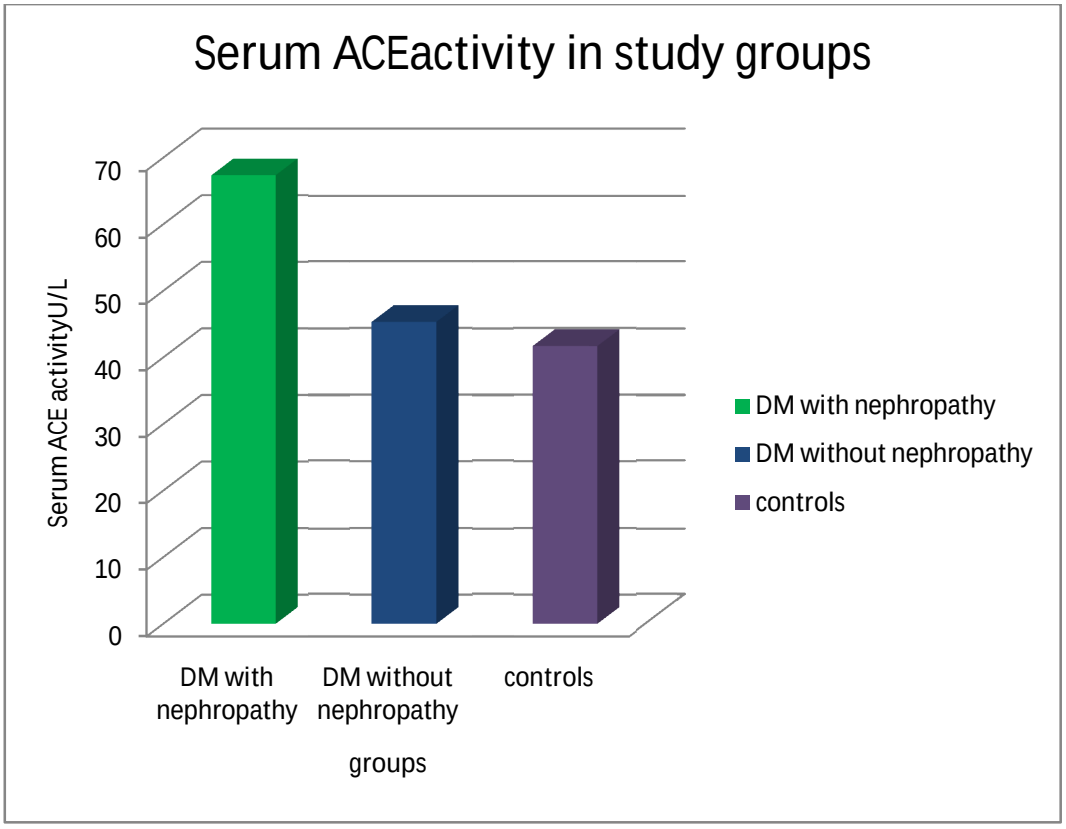


**TABLE-5**

## Serum ACE activity in study groups

| | Group 1A -DM with nephropathy | Group 1B-DM without nephropathy | Control | P value |
|---|---|---|---|---|
| **Serum ACE activity U/L (Normal range is 8-52)** | **67.40 ±22.478** | **45.30 ± 21.324** | **41.67 ± 17.440** | **0.000-S** |



- The ACE activity of Group 1B and Control are within the normal range but that of Group 1A (diabetic nephropathy) is well elevated above upper limit of normal.

**Table 6**

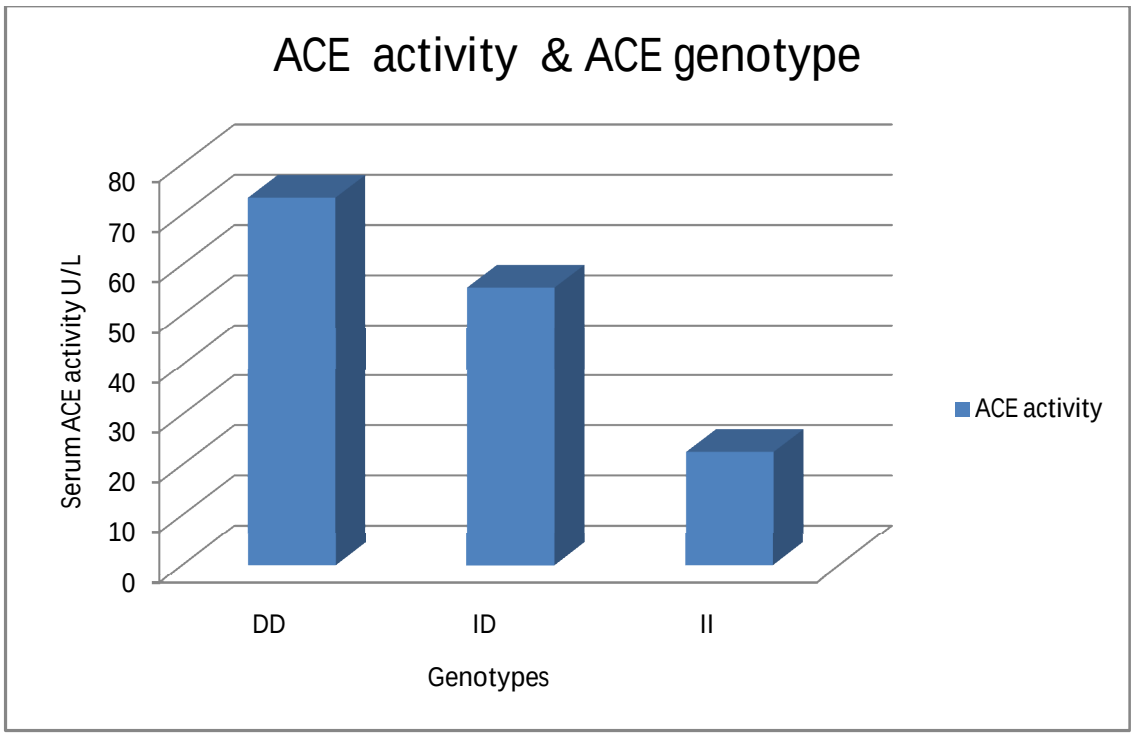
Shows the association of ACE genotype with the phenotype(ACE activity)

- ACE genotype and its phenotype(ACE activity) were compared.
- It is observed that serum ACE activity is elevated in DD genotype
- Highest(73U/L) level of ACE activity in DD genotype, lowest(22U/L) in II genotype and intermediate(55U/L) in ID genotype .
- P value =0.000 which is highly significant statistically.

**TABLE-6**

## Genotype and Phenotype(ACE activity)

| Genotype | ACE activity(U/L)<br>Normal range : 8-52 | P value |
|---|---|---|
| **DD** | 73.36±14.54 | **0.000-S** |
| **ID** | 55.49±5.65 | |
| **II** | 22.61±8.65 | |



# DISCUSSION

# DISCUSSION

Long term type 2 diabetes is a major and important cause of nephropathy. Despite well known risk factors large variation in prevalence among different diabetic populations and clustering of diabetic nephropathy in families[5,6] suggest genetic factor involvement. renin–angiotensin–aldosterone system(RAAS) plays a central role in the regulation of blood pressure, sodium metabolism and renal hemodynamics, with its actions mediated primarily by angiotensin II. The gene encoding components of the RAAS,especially ACE gene is extensively studied.

In our study we found that the urine microalbumin was elevated but blood urea and serum creatinine were within the normal limits in diabetic nephropathy patients. This indicates that these nephropathy patients are in their early stage disease.

From our study we found a more frequent association of DD genotype of ACE gene in diabetic nephropathy group patients (56.7%) when compared to controls and type 2 diabetics without nephropathy. The Odds ratio between diabetics with nephropathy and diabetics without nephropathy was found to be 5 in our study. This is similar to

studies done by Viswanathan et al, and Bhavani et al, who found positive association between the D allele of the ACE gene polymorphism and diabetic nephropathy in South Indian type 2 diabetic patients. Whereas studies done in North Indian population by Ajay kumar et al, and Prasad P.et al found there was no relation between ACE gene polymorphism and development of diabetic nephropathy. It is reported that the D polymorphism of ACE gene is associated with susceptibility to diabetic nephropathy with more marked association among type 2 diabetic Asians than in Caucasians[130]. Other studies have shown that the deletion polymorphism can affect both the risk and progression of diabetic nephropathy whereas Schmidt et al. demonstrated that the deletion polymorphism may act on progression of but not susceptibility to diabetic nephropathy[131]. Several Japanese studies had also found the D allele to be an independent risk factor for diabetic nephropathy in type 2 diabetic patients[132-134]. The odds ratio noted in our study for the association of $D^+$ allele with nephropathy was found to be 4. In our study we found that ACE activity was elevated in the diabetic nephropathy group with the mean value of 67 U/L while that of diabetics without nephropathy group it was 45 U/L and that of controls was 41U/L. The difference was found to be statistically significant(P value=0.000) between diabetic nephropathy

cases and controls. The ACE gene polymorphism was first reported by Rigat et al in a study that addressed the role of the ACE gene in the genetic control of plasma ACE levels. The ACE DD genotype is associated with increased circulating ACE levels, which are generally two times as high as those found for II genotypes; ID heterozygotes are associated with intermediate ACE levels[18]. This relationship of D allele and enzymatic levels, originally reported by Rigat et al, was repeatedly confirmed by other studies, for both circulating and cellular ACE[100,101,135]. ACE gene polymorphism determines the serum and tissue ACE activity in subjects with DD polymorphism (Malik et al., 1997). However, because the ACE I/D polymorphism is intronic, the mechanism of ACE overexpression in subjects with DD genotype is unclear. It is thought to be in linkage disequilibrium with a functional mutation in the gene[19].

Highest(73U/L) level of ACE activity in DD genotype, lowest(22U/L) in II genotype and intermediate(55U/L) in ID genotype was observed in our study with significant statistical difference (p value=0.000). These findings are in concordance with other studies.

# CONCLUSION

# CONCLUSION

This study was conducted to find out the ACE gene insertion(I)/deletion(D) polymorphism among diabetics with nephropathy and without nephropathy. 30 cases of diabetics with nephropathy were compared with 30 diabetics without nephropathy.

From our study we found that :

1. Type 2 diabetics with nephropathy had a higher frequency of ACE DD genotype compared to diabetics without nephropathy and controls.

2. Serum ACE activity is significantly elevated in Diabetic nephropathy patients which is responsible for the nephropathic changes.

3. Serum ACE activity in diabetics without nephropathy is within the reference range and is comparable with the control group.

4. The level of Serum ACE activity was highest in DD genotype, lowest in II genotype and intermediate in ID genotype and hence DD genotype is strongly associated with nephropathy.

5. DD genotype is an independant risk factor for the development of nephropathy.

# FUTURE PROSPECTS OF THE STUDY

# SCOPE FOR FURTHER STUDY

Angiotensin converting enzyme(ACE) is known to catalyse the conversion of Angiotensinogen to Angiotensin II which is responsible for efferent arteriolar constriction. Hence elevated levels of ACE are responsible for the pathogenesis of glomerulosclerosis leading to nephropathy. The ACE gene polymorphism has been widely studied as candidate gene in the development of diabetic nephropathy.

Studies have reported conflicting data regarding ACE/ID polymorphism and the individual antiproteinuric response to ACE inhibition. It is also reported that patients with ACE DD genotype showed poor response to ACE inhibition. The beneficial effects of losartan were greatest in the ACE/DD group and intermediate in the ID group suggesting a quantitative interaction between losartan treatment and ACE/ID genotype on progression of renal disease providing renoprotection in all ethnic groups[136].

In our study we have not confirmed the DD genotypes using insertion specific primers to rule out the possibility of mistyping[137] due to amplification of D allele and the DD positive cases have to be confirmed again by using another insertion specific primer.

Future prospects:

1. ACE gene polymorphism screening can be done in families with type 2 diabetes for risk of development of nephropathy.

2. ACE gene polymorphism screening can be done to monitor progression of severity of nephropathy in diabetics.

3. Screening for personalising treatment with ACE inhibitors or Angiotensin receptor blockers.

4. Long term renoprotective effects with angiotensin receptor blockers have to be evaluated.

5. ACE DD genotype can be confirmed with insertion specific primers to rule out the possibility of mistyping due to amplification of D allele.

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# INSTITUTIONAL ETHICS COMMITTEE
# MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone :044 25305301
Fax: 044 25363970

## CERTIFICATE OF APPROVAL

To
Dr. K. Menaka Shanthi
PG in MD Biochemistry
Madras Medical College, Chennai-3,

Dear Dr. K. Menaka Shanthi

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled " Association of Angiotensin converting Enzyme gene insertion / deletion polymorphism in type 2 diabetic with nephropathy" No. 21032011.

The Following Members of Ethics committee were present in the Meeting held on 17.03.2011 conducted at Madras Medical College, Chennai -3

| | |
|---|---|
| 1. Prof. S.K. Rajan MD | -- Chairperson |
| 2. Prof. V. Kangasabai .MD<br>Dean, Madras Medical College, Chennai -3 | -- Deputy Chairman |
| 3. Prof. A. Sundaram. MD<br>Vice Principal, Madras Medical College, Chennai -3 | -- Member Secretary |
| 4. Prof. R. Nandhini MD<br>Director, Institute of Pharmacology, MMC, Ch-3 | -- Member |
| 5. Prof. C. Rajendiran MD<br>Director , Institute of Internal Medicine, MMC, Ch-3 | -- Member |
| 6. Prof. Geetha Subramanian MD. DM<br>Prof. & Head, Dept, of cardiology, MMC, Ch-3 | -- Member |
| 7. Prof.. Mohammed Ali MD DM<br>Prof & Head, Dept. of MGE, MMC, Ch-3 | -- Member |
| 8. Thiru . A. Ulaganathan<br>Administrative Officer, MMC, Ch-3 | -- Layperson |
| 9. Thiru. S. Govindasamy BA BL | -- Lawyer |
| 10. Tmt. Arnold Saulina | -- Social Scientist |

We approve the proposal to be conducted in its presented form.

Sd/ chairman & Other Members

The Institutional Ethics committee expects to be informed about the progress of the study and SAE occurring in the course of the study , any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report.

Member Secretary, Ethics Committee